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II. Benzidine

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II. Benzidine

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This report is a review of the scientific literature on the biological and environmental effects of benzidine. Included in the review are a general summary and a comprehensive discussion of the following topics as related to benzidine and specific benzidine compounds: physical and chemical properties; occurrence; synthesis and use; analytical methodology; biological aspects in microorganisms, plants, wild and domestic animals, and humans; distribution, mobility, and persistence in the environment; assessment of present and potential health and environmental hazards; and review of standards and governmental regulations. More than 200 references are cited.

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FOREWORD

A vast amount of published material is accumulating as numerous research investigations are conducted to develop a data base on the adverse effects of environmental pollution. As this information is amassed, it becomes continually more critical to focus on pertinent, well-designed studies. Research data must be summarized and interpreted in order to adequately evaluate the potential hazards of these substances to ecosystems and ultimately to public health. The Reviews of the Environmental Effects of Pollutants (REEPs) series represents an extensive compilation of relevant research and forms an up-to-date compendium of the environmental effect data on selected pollutants.

Reviews of the Environmental Effects of Pollutants: II. Benzidine includes information on chemical and physical properties; pertinent analytical techniques; transport processes to the environment and subsequent distribution and deposition; impact on microorganisms, plants, and wildlife; toxicologic data in experimental animals including metabolism, toxicity, mutagenicity, teratogenicity, and carcinogenicity; and an assessment of its health effects in man. The large volume of factual information presented in this document is summarized and interpreted in the final chapter, "Environmental Assessment," which presents an overall evaluation of the potential hazard resulting from present concentrations of benzidine in the environment.

The REEPs are intended to serve various technical and administrative personnel within the Agency in the decision-making processes, i.e., in the development of criteria documents and environmental standards, and for other regulatory actions. The breadth of these documents makes them a useful resource for public health personnel, environmental specialists, and control officers. Upon request these documents will be made available to any interested individuals or firms, both in and out of the government. Depending on the supply, the document can be obtained directly by writing to:

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ABSTRACT

This study is a comprehensive, multidisciplinary review of the health and environmental effects of benzidine and specific benzidine derivatives. More than 200 references are cited.

Benzidine and its congeners are used in industry primarily for the synthesis of azo dyes. Available data indicate that U.S. production and importation of benzidine and benzidine compounds may exceed 3.5 million kilograms per year. Benzidine and its derivatives are not naturally occurring compounds and are not thought to be generally dispersed in the environment; however, little information is available for documentation.

In most animal species tested, the greatest damage from benzidine exposure occurred to the liver, kidney, and urinary bladder. Following administration of sublethal doses, tumors have developed in almost all species tested. Little information is available on the effects of benzidine on plants or on domestic and wild animals.

Epidemiological evidence leaves no doubt that exposure to benzidine can lead to bladder cancer in humans. An exact quantitative relationship between the degree of exposure to benzidine, the length of the exposure period, and the incidence of bladder cancer has not been established. Benzidine compounds are believed to become active carcinogens only after metabolic alterations. Since 1974, when the Occupational Safety and Health Administration of the U.S. Department of Labor instituted more stringent industrial standards, the risk of cancer from industrial exposure to benzidine has been substantially reduced. There is no evidence that benzidine or its congeners act as human teratogens; however, some tests suggest that these compounds may be mutagenic. Benzidine compounds are also known to cause dermatitis, cystitis, and hematuria.

This report was submitted in partial fulfillment of Interagency Agreement No. D5-0403 between the Department of Energy and the U.S. Environmental Protection Agency. The draft report was submitted for review June 1976. The final report was completed in October 1977.

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SECTION 1

SUMMARY

1.1 INTRODUCTION

Benzidine is an aromatic amine that does not occur naturally; it was first prepared by Zinin in 1845 and is now used in the synthesis of commercial dyes. 3,3'-Dichlorobenzidine, 3,3'-dimethylbenzidine, and 3,3'-dimethoxybenzidine are benzidine derivatives widely used in the United States and therefore are included in this discussion. Two benzidine congeners, 4-aminobiphenyl and 4-nitrobiphenyl, are discussed because of structural and chemical similarities to benzidine.

1.2 BENZIDINE CHEMISTRY

1.2.1 Physical Properties

Benzidine is a colorless, crystalline compound with a molecular weight of 184.23, a density of 1.250 (20/4°C), a melting point of 115°C to 120°C (slow heating) or 128°C (fast heating), and a boiling point of 400°C. It is soluble in alcohol and ether and is slightly soluble in water. 3,3'-Dichlorobenzidine, a colorless, crystalline compound, has a molecular weight of 253.1 and a melting point of 132°C to 133°C. It is soluble in ethanol, benzene, and glacial acetic acid, is slightly soluble in dilute hydrochloric acid, and is almost insoluble in cold water. 3,3'-Dimethylbenzidine is a white to reddish crystalline compound which has a molecular weight of 212.28 and a melting point of 131°C to 132°C; it is highly soluble in ethanol, ethyl ether, and acetone and is slightly soluble in water. 3,3'-Dimethoxybenzidine, a colorless, crystalline compound that turns violet on standing, has a molecular weight of 244.3 and a melting point of 137°C to 138°C. It is soluble in ethanol, ethyl ether, acetone, benzene, and chloroform and is poorly soluble in water. 4-Aminobiphenyl is also a colorless, crystalline compound that darkens on oxidation. The molecular weight is 169.2, the melting point is 53°C to 54°C, and the boiling point is 191°C. It is soluble in lipids, hot water, and nonpolar solvents and is slightly soluble in cold water. The compound 4-nitrobiphenyl occurs as white to yellow needles. It has a molecular weight of 199.2, a boiling point of 340°C, and a melting point of 114°C. 4-Nitrobiphenyl is soluble in benzene, chloroform, and ether, is slightly soluble in cold alcohol, and is almost insoluble in water.

1.2.2 Chemical Properties

Benzidine is both a primary aromatic amine and a diphenyl derivative; it undergoes chemical reactions which are characteristic for each class of compounds as well as some which are peculiar to it alone (Section 2.2.1.2). Benzidine forms stable salts with mineral acids and complexes with a large number of metallic salts. It is sensitive to oxidation and can be readily acetylated, alkylated, and diazotized. The acetylated product is readily nitrated and halogenated. The chemical reaction leading to the diazotized product has commercial value since this product couples with many different aromatic amines and phenols to yield a wide variety of azo dyes.

The benzidine derivatives 3,3'-dichlorobenzidine, 3,3'-dimethylbenzidine, 3,3'-dimethoxybenzidine, and 4-aminobiphenyl (Sections 2.2.2.1, 2.2.3.1, 2.2.4.1, and 2.2.5.1) undergo chemical reactions similar to those displayed by benzidine. They can be acetylated, alkylated, and diazotized. The latter reaction is commercially important as the first step in their use in the synthesis of azo dyes. Because 4-nitrobiphenyl (Section 2.2.6.1) contains no reactive amino group, its chemical reactions differ. However, it is readily reduced to 4-aminobiphenyl.

1.2.3 Biochemical Reactions

In a manner similar to the biochemical reactions of aromatic amines in general, benzidine is conjugated through the functional amino groups (usually only one group is conjugated) and hydroxylated in the *ortho* position with respect to the amine. Hydroxylation of benzidine requires mediation by an enzyme system consisting of a hepatic microsomal enzyme, oxygen, and reduced nicotinamide adenine dinucleotide phosphate. The hydroxy groups of the hydroxybenzidine thus formed may be conjugated with sulfate and with glucuronic acid through mediation of other required enzyme systems.

Less attention has been given to the biochemical reactions that occur with benzidine derivatives, but, with the exception of 3,3'-dichlorobenzidine, the reactions thought to occur are similar to those for benzidine — namely, acetylation, sulfation, and orthohydroxylation. Because of the in vivo stability of the chlorine atom linkage to the benzidine molecule, 3,3'-dichlorobenzidine apparently does not form 3-hydroxy products. This compound appears to be relatively stable in vivo.

Although the process is still somewhat speculative, it appears that the azo dyes prepared from benzidine and its derivatives may be reduced in vivo to release the parent materials. A recent report on this possible reduction is discussed in Section 2.2.1.4.

1.2.4 Methods of Analysis

Several methods of analysis of benzidine, its derivatives, and their metabolites are used for qualitative and quantitative determinations in environmental and biological media. These methods are gas-liquid chromatography, thin-layer chromatography, spectrophotometry (chloramine-T), spectrophotometry (diazo dye), and spectrophotofluorometry (Section 2.3.2). None of these is officially sanctioned for determining low levels of benzidine in environmental or biological samples, but several appear adequate for assessment of gross amounts of aromatic amines. Gas-liquid chromatography seems to be the most promising method of benzidine analysis because of its high degree of sensitivity, selectivity, versatility, and speed. This is the only available method which easily determines benzidine in the presence of its derivatives.

The oldest and best developed analytical methods are the diazo and chloramine-T spectrophotometric methods. Recent refinements allow sensitivity in the parts-per-billion range by instrumentation available in most laboratories. However, neither method can be used to distinguish between benzidine and benzidine derivatives in mixed samples. Furthermore, the diazo method is responsive to aromatic amines in general, while the chloramine-T method is photosensitive. Nevertheless, the U.S. Environmental Protection Agency has designated the chloramine-T method as the interim method for determining benzidine.

The thin-layer chromatographic method is simple to perform and frequently is used qualitatively for separating and identifying benzidine, benzidine derivatives, and metabolites. Although it can be used quantitatively, it is relatively insensitive.

Spectrophotofluorometry has had only limited use with benzidine compounds. The method combines inherent sensitivity with simplicity and rapidity. However, the fluorescent spectra produced by benzidine and its derivatives tend to overlap and vary greatly in their intensity, thus preventing the method from being used to quantitatively distinguish such compounds in mixed samples.

1.3 USES AND PRODUCTION

Benzidine and its salts are used principally in the manufacturing of dyestuffs based on the coupling of tetrazotized benzidine with phenols and aromatic amines. At least 250 commercial dyes are based on benzidine. Many of these dyes are economically important because of their ability to dye cotton without the use of mordants. In analytical chemistry, benzidine is used to detect inorganic ions (Section 2.2.1.3), as a chromogenic spray reagent in chlorinated organic pesticide thin-layer chromatography, and as a reagent for determining hydrogen peroxide, nicotine, sugars, occult blood, and bacterial cytochromes. Benzidine is also used as a hardener for polyurethane and as an agent to reveal bank check alteration. Production in the United States was 680.9 metric tons in 1972.

The benzidine derivatives 3,3'-dimethylbenzidine, 3,3'-dimethoxybenzidine, and 3,3'-dichlorobenzidine are used in the manufacture of dyestuffs. These dyestuffs are used to color plastics, lacquer, paint, rubber, leather, paper, and textiles. They are used in the detection and determination of various inorganic ions. 3,3'-Dichlorobenzidine and 3,3'-dimethoxybenzidine are used as curing agents for polyurethane elastomers; the latter of these two compounds is also used in the manufacturing of the diisocyanate compound for isocyanate-based adhesive systems. 4-Aminobiphenyl has been used as an antioxidant in rubber. 4-Nitrobiphenyl is an intermediate in the manufacture of 4-aminobiphenyl.

Production in the United States of benzidine, 3,3'-dichlorobenzidine, 3,3'-dimethylbenzidine, and 3,3'-dimethoxybenzidine is believed to amount to many millions of kilograms annually. Benzidine and 3,3'-dichlorobenzidine are produced in greatest quantities. Large-scale production of 4-aminobiphenyl and of 4-nitrobiphenyl in the United States is believed to have ceased following the general recognition in 1955 of the carcinogenicity of the former compound; however, small amounts are still synthesized for research and pilot plant use.

1.4 ENVIRONMENTAL DISTRIBUTION AND FATE

Since the melting points of benzidine and its derivatives are above ambient environmental temperatures, these compounds exist in the undissolved state as aerosols, dusts, or crystals and in the dissolved state in aqueous or organic solutions.

1.4.1 Distribution in Air

At one time, benzidine and its derivatives were manufactured and used in open systems which permitted loss of the compounds to the atmosphere, the worker, and the work site. In some operations, average concentrations of 2.5 to 17.6 mg/m^3 of air were observed. Large-scale manufacturers are now believed to utilize closed systems which prevent this hazard, except possibly during cleaning of the closed-system equipment. At present, small-scale manufacturing may be proceeding without the use of closed systems.

The use by the general public of benzidine yellow pigments in pressurized containers of lacquers and paints presents an exposure of currently unknown potential and hazard. This pigment is derived from 3,3'-dichlorobenzidine.

1.4.2 Distribution in Water

In the Sumida River of Japan, benzidine has been measured downstream from dye factories. In the United States, benzidine and its derivatives have been detected in manufacturing and dye factory wastes but not in the drainage areas into which these wastes feed; however, few attempts at actual measurements seem to have been made. Lack of detection of these compounds is thought to result from degradation, mostly before the release of the wastes, and from dilution of residual concentrations to below the detection limit. Unfortunately, the chloramine-T method of detection used evidently will measure only the parent benzidine compounds and may therefore have failed to measure the possible presence of slightly degraded products, including those of carcinogenic potential. Newer analytical methods now under study should be more versatile (Section 1.2.4).

1.4.3 Distribution in Soil

No data were found concerning the distribution of benzidine compounds in soil. Benzidine has been shown to be adsorbed to some clays and, because of its physical properties, is probably immobilized rapidly in soils and sediments.

1.4.4 Mobility and Persistence

Benzidine is thought to be volatile and soluble enough to have the potential for wide dispersion. According to several investigators, it probably persists for a time in the environment. The principal reaction of benzidine in air and water is believed to be oxidative degradation by free radical, enzymatic, or photochemical processes, but actual data on reaction rates are not available. Half-life reaction rates have been estimated to be 1 to 60 days in air and 100 days in water for benzidine and 3,3'-dichlorobenzidine. Under experimental water waste treatment conditions, air oxidation of benzidine proceeded readily, and there was also some evidence of biological oxidation.

1.4.5 Food Chain Accumulation

Since benzidine and its derivatives are soluble in many organics, movement of these compounds may take place within the food chain. Accumulation is expected to occur, and recent laboratory findings indicated that bluegill fish concentrated ^{14}C-benzidine to a level 44 times that present in water.

1.5 BIOLOGICAL ASPECTS IN MICROORGANISMS, PLANTS, AND WILD AND DOMESTIC ANIMALS

Very little information is available concerning the metabolism and effects of benzidine and its derivatives in these classes of organisms.

1.5.1 Microorganisms

The metabolism of benzidine by microorganisms has received little attention. It is known, however, that benzidine-based azo dyes are reduced to benzidine by certain bacteria.

Little is known about the toxicity of benzidine and its derivatives to microorganisms. At low concentrations, benzidine appears to be oxidized by microorganisms present in the activated sludge of waste treatment plants, but at higher concentrations it may inhibit these microorganisms and prevent general biooxidation of the organic material present. Benzidine and some of its derivatives have been shown to be mutagenic to strains of *Salmonella typhimurium* during testing by an assay method developed for measuring the mutagenic and carcinogenic potential of a wide variety of chemicals.

The benzidine-based azo dye Congo Red has been used as a bacteriostatic agent in the selective culture of the tubercle bacillus and as an indicator dye in the culture of other species of bacteria. Congo Red and other azo dyes have been shown to have antiviral activity and to inhibit phage production by one species of bacteria.

1.5.2 Plants

No data are available on benzidine metabolism or on effects from benzidine exposure in higher plants. Benzidine has not received much attention in this area since it is not a natural environmental constituent and is not believed to be widely dispersed from industrial sources. Benzidine (0.3 mg/liter) stimulates growth of *Antithamnion plumula* (red algae). It is oxidized by peroxidases from several plant species, including horseradish and green algae.

1.5.3 Wild and Domestic Animals

Information pertaining to wild and domestic animals and benzidine compounds does not exist except for laboratory research on a few species under experimental conditions. Benzidine has been shown to be toxic to the red shiner and fathead minnow at TL_{50} values of 2.5 mg/liter and 20 mg/liter, respectively, and to cause liver damage, including hyperplasia, in guppies fed a diet containing 30 mg benzidine per 100 g of dry feed. When injected into eggs, benzidine caused morphological abnormalities in immature chick embryos. Other research findings with the more usual laboratory animals (e.g., rat, mouse, and dog) are summarized in Section 1.6 since such data provide useful comparative information for studying the metabolism and toxicity of benzidine compounds in humans.

Because benzidine and its derivatives may possibly find their way into the open environment, much more research is needed to determine species differences in the uptake, accumulation, metabolism, and excretion of these compounds and in the toxic effects that may occur through exposure.

1.6 BIOLOGICAL ASPECTS IN HUMANS

Benzidine exposure in humans is primarily an industrial problem. Because benzidine and its derivatives are not natural constituents of the environment and the general population is therefore not exposed to these chemicals, information concerning human exposure is restricted to industrial exposure.

1.6.1 Metabolism

1.6.1.1 Uptake, Distribution, and Accumulation — Benzidine compounds enter the body through the respiratory tract, by penetration of the skin, or by ingestion. The inhalation of these compounds is believed to be minimized by the use of closed systems during manufacture and use (Section 1.4.1); a concentration of 0.02 mg benzidine per cubic meter of air has been suggested as a safe level of exposure. Penetration through the skin is believed to be the most important avenue of uptake. Penetration is increased at elevated air temperatures and humidities which cause sweaty skin. Some compounds penetrate more readily than others (benzidine > 3,3'-dimethoxybenzidine > 3,3'-dichlorobenzidine). Ingestion via contaminated food is not generally thought to be an important route of uptake, but ingestion is believed to be responsible for some cancer induction among Japanese kimono painters because of their habit of moistening brushes that have been dipped in benzidine-based azo dyes with their lips.

Information is lacking on the distribution or accumulation of benzidine and its derivatives in human tissues. Indirect evidence afforded by repeated measurements of benzidine in urine samples from exposed workers indicated a lack of any appreciable accumulation.

Data collected from experimental animals is more complete. In the rat, dog, and monkey, administered concentrations of benzidine and 3,3'-dichlorobenzidine were distributed within hours to various tissues. Prior to the elimination of these compounds, which was virtually complete in one week, concentrations were noticeably higher in liver and lung tissues than in other tissues. In similar testing, 3,3'-dimethylbenzidine administered to the rat was found in highest concentration in the Zymbal's gland. The liver and Zymbal's gland tissues of the rat are among those tissues most prone to tumor induction by benzidine and its derivatives, perhaps because of the tendency of these compounds to concentrate within such tissues (Section 6.3.2.2).

There is indirect evidence for possible placental transfer of benzidine or its derivatives since some of these compounds have been found to affect embryonic kidney tissue following subcutaneous injection of pregnant mice and, later, explantation and culture of the embryonic tissue (Section 6.3.2.1.4).

Although there is no evidence of any significant bioaccumulation of benzidine or its derivatives in humans or in the usual experimental animals, it has been reported recently that edible portions of bluegill fish contained a 44-fold increase in residues of ^{14}C-benzidine over those present in water during a radiometric study.

1.6.1.2 Biotransformation and Elimination — Benzidine and its derivatives are biotransformed in humans and in experimental animals. The metabolites as well as the parent materials are eliminated via the urine, bile, and feces. Some of the metabolites produced are thought to be the actual carcinogenic forms of these compounds (Sections 2.2 and 6.2.3).

Humans metabolize benzidine primarily to 3-hydroxybenzidine and secondarily to monoacetylbenzidine, diacetylbenzidine, and N-hydroxy acetyl amino benzidine. These compounds, as well as benzidine, are eliminated largely through urinary excretion. Urinary excretion in exposed workers appears to vary directly and in proportion to the amount of benzidine absorbed. Thus, a safe air concentration of benzidine has been suggested as 0.2 mg or less of benzidine per cubic meter of air since this amount, inhaled over an 8-hr period, is calculated to lead to a urinary output not exceeding 0.26 mg benzidine per liter of urine. 3,3'-Dimethylbenzidine appears to be biotransformed and eliminated in a manner similar to that of benzidine. Workers exposed to benzidine yellow have reportedly excreted 3,3'-dichlorobenzidine in their urine, probably because of the metabolic reduction of the dye and release of this benzidine derivative.

The metabolites of benzidine found in humans are also found in some experimental animals, along with additional products resulting from sulfate and glucuronide conjugations. Species differences occur among the test animals in the type of metabolites produced and in the main routes of elimination. In comparative tests, the dog was unable to acetylate benzidine, although this metabolic reaction occurred in the mouse, rat, rabbit, and guinea pig. Metabolites of benzidine with available amino groups were produced in larger amounts by the dog than by the rabbit. Benzidine was found to be eliminated largely through the urine of the dog, through the feces of the rat, and in about equal amounts through the urine and feces of the monkey. Benzidine metabolites found in bile appear to be similar to those found in urine but are present in only about one-third the amount found in urine.

Monkeys are capable of metabolically reducing a number of benzidine-based azo dyes and thereby freeing the benzidine molecule. Substantial amounts of benzidine and of monoacetylbenzidine were found in the urine of the animals following single oral doses of the dyes.

The metabolism and excretion of benzidine derivatives have been less extensively studied in experimental animals. In one experiment, administered doses of 3,3'-dichlorobenzidine in dogs were only partially accounted for by measured urinary products, some of which were the nonmetabolized parent materials. No metabolites of 3,3'-dichlorobenzidine were found, suggesting that this compound, by virtue of the chlorine substitutions at the 3,3' positions, is more inert to biotransformation than are the methyl or methoxy substitutions. A separate study showed that 3,3'-dichlorobenzidine in dogs is excreted in greater quantity by the feces than by the urine, in direct contrast to the excretion pattern of benzidine. This finding indicated that the route of excretion was altered by substitution of the benzidine molecule in the 3,3' positions.

Dogs and monkeys fed 4-aminobiphenyl excreted N-hydroxy products of this compound more rapidly in their urine than they excreted the parent compound. The urinary yield of a 3-sulfuric acid conjugate of 4-aminobiphenyl varied widely among five species of animals, ranging from 0% in the guinea pig to 25% to 40% in the dog.

1.6.2 Effects

1.6.2.1 Physiological Effects — A possible relationship was found during in vitro studies between potential carcinogenicity of benzidine and its derivatives and their ability to reduce cytochrome *c*. 4-Aminobiphenyl, considered to be strongly carcinogenic, did not reduce cytochrome *c*, whereas 3,3'-diaminobenzidine, considered to be only weakly carcinogenic, did reduce this enzyme. Aminobiphenyls, which are easily oxidized by mitochondrial systems, may be detoxified in this manner and excreted in combination with sulfuric or amino acids.

Exposure to benzidine has been found to alter enzyme activity in vivo. The activity of β-glucuronidase was higher in the urine of exposed workers than in nonexposed workers. Cessation of exposure caused a decrease in activity to a level more nearly normal. Blood phenolase activity in rabbits was decreased by benzidine but not by 3,3'-dimethylbenzidine. Catalase and peroxidase activity was decreased in rats by benzidine.

1.6.2.2 Toxic Effects — In addition to their carcinogenic potential, benzidine compounds are known to cause dermatitis, cystitis, and hematuria in humans and a number of poisoning symptoms, including glomerulonephritis, in laboratory animals (Section 6.3.2). Dermatitis has been reported among workers in dye manufacturing plants. Benzidine and benzidine-based dyes have been implicated. Benzidine and 3,3'-dimethylbenzidine have caused dermatitis among laboratory-exposed chemists. Individuals appear to vary in sensitivity to these compounds.

Toxic effects to experimental animals are varied. For example, mice fed a daily diet of benzidine lost weight in proportion to the amount of compound ingested, but the weight of the spleen and thymus increased. Morphological changes included a cloudy swelling of the liver, vascuolar degeneration of renal tubules, and hyperplasia of the myeloid elements in bone marrow and of the lymphoid cells in the spleen and thymic cortex. Several investigators have reported glomerulonephritis in rats exposed to N,N'-diacetylbenzidine. The nephrotic syndrome induced resembles glomerulonephritis in humans. At the high dosage rates applied to test animals in carcinogenicity studies, liver pathology is severe and may lead to death prior to any tumor development.

There is convincing evidence that benzidine and certain other aromatic amines are responsible for the high rate of occurrence of bladder cancer among occupationally exposed persons. It is generally accepted that metabolites of these aromatic amines are the actual carcinogens. Evidence suggests that N-hydroxylation of the parent amine produces the precarcinogen and that the sulfate or glucuronide conjugates are the carcinogenically active forms in vivo. The latter metabolites appear to have an affinity to and exert a carcinogenic action on certain target organs. Cancer induction in humans by benzidine appears to be limited to the bladder.

Since benzidine and its derivatives are not naturally occuring chemicals and are not thought to be dispersed in the environment, the thrust of studies on their carcinogenicity has been directed at groups of occupationally exposed workers in manufacturing and user industries of various countries. Tumor development in these workers has been reported following exposures to benzidine ranging from 2 to 16 years and averaging 8 years. The latent period between first exposure and tumor induction ranges from 8 to 32 years and averages 18 years. Thus, workers who have been exposed to benzidine for as little as two years have developed bladder cancer several years later. The degree of exposure and the number of years of survival beyond the exposure period are thought to influence the rate of occurrence of bladder tumors. High rates of occurrence averaging 11% to 50% of the exposed workers have been reported from benzidine manufacturing plants, dyestuff factories, and coal tar dye plants. Awareness of this hazard has prompted improved work conditions and work habits, which have resulted in reduced exposure. The introduction of closed systems in the synthesis of benzidine has greatly reduced atmospheric contamination. Unfortunately, there is a general lack of information on actual exposures encountered in various plants. More data should be collected and made available on air concentrations, on respiratory and dermal absorption, and on the physical conditions and health of the workers.

In some earlier studies on the rate of occurrence of bladder cancer in manufacturing and dyestuff plants, the role of benzidine in the induction of the cancer was confused by simultaneous exposures to other chemicals, particularly to β-naphthylamine, which is considered to be a potent carcinogen. Later comparative studies indicated that such dual exposures may significantly increase the risk of bladder cancer induction. One study, for example, showed an increase from 21.3% among workers exposed only to benzidine to 45.5% among workers exposed additionally to β-naphthylamine.

In humans, benzidine has been implicated only in the induction of bladder cancer. In contrast, it induces several different types of cancer in experimental animals. The evidence of cancer induction by benzidine and its derivatives suggests that induction by these compounds depends on both species and compound.

3,3'-Dichlorobenzidine, unlike benzidine, has not been reported to have produced bladder tumors in humans, but it has been implicated recently in the occurrence among exposed workers of tumors of the lung, bone marrow, rectum, sigmoid colon, prostate, and breast muscle. These workers were exposed for 35 years. Since dichlorobenzidine is nonvolatile and poorly absorbed by the skin, respiratory and dermal intake can be expected to be less than that which would occur with benzidine for an equivalent period. Experimentally, a variety of tumors have been induced by dichlorobenzidine in rats and mice, including bladder tumors in rats. It is possible that this compound can induce bladder cancer in humans, but only after a latent period longer than that for benzidine.

There is no evidence that 3,3'-dimethylbenzidine or 3,3'-dimethoxybenzidine induce any type of cancer in humans. However, both compounds have been found to be carcinogenic in rats and have induced a variety of tumors. They must therefore be considered as potentially carcinogenic in humans.

4-Aminobiphenyl is considered to be a potent carcinogen in humans. Bladder tumors have developed in workers following as little as 133 days of exposure as well as after more extended exposure periods ranging up to 19 years. The latent period appears to range from 5 to 19 years. This compound causes a variety of tumors in experimental animals, including bladder cancer. However, its production and use in the United States are believed to have been greatly reduced by the early recognition of its carcinogenicity.

1.7 THE PROBLEM

Benzidine presents a serious carcinogenic hazard to exposed industrial workers because of its association with the high rate of occurrence of bladder cancer among these workers. It is a potent carcinogen in experimental animals. Derivatives of benzidine also cause cancer in experimental animals, but only 4-aminobiphenyl has been associated with bladder cancer in humans; usage of this compound is thought to have decreased markedly in recent years. 3,3'-Dichlorobenzidine has recently been implicated in causing cancer other than bladder cancer in humans, but no reports have implicated either 3,3'-dimethylbenzidine (diorthotolidine) or 3,3'-dimethoxybenzidine (dianisidine). Nevertheless, these derivatives must be viewed as potentially hazardous to humans. The use of closed systems and better hygiene by workers have resulted in a decrease in the level of exposure to these compounds and thereby have reduced the hazard involved.

Benzidine and its derivatives are not naturally occurring compounds and are not thought to be dispersed in the environment, although documentation on this is lacking. Judging from their toxic effects to research animals, these compounds, if present in the environment, can be expected to be hazardous to wildlife. Recent laboratory information indicating appreciable bioaccumulation in one species of fish increases the hazard potential.

1.8 CONCLUSIONS

1. Benzidine and its derivatives are not naturally occurring compounds and are probably not found in the general environment, although adequate documentation is lacking.

2. Analysis of benzidine and its derivatives can be performed best by use of gas-liquid chromatography or spectrofluorometry; these methods provide needed sensitivity, selectivity, and versatility.

3. The chloramine-T method of analysis is designated as the interim method of benzidine analysis by the U.S. Environmental Protection Agency, but, unfortunately, it does not adequately identify or distinguish between benzidine and its derivatives and the important metabolites.

4. Benzidine and the derivatives 3,3'-dichlorobenzidine, 3,3'-dimethylbenzidine, and 3,3'-dimethoxybenzidine are manufactured in the United States by three major firms which employ closed systems to reduce air contamination and exposure by the workers. There is reason to believe that some manufacturing, including limited production of 4-aminobiphenyl, is being undertaken by smaller firms without the use of the closed-system approach.

5. Benzidine, 3,3'-dichlorobenzidine, 3,3'-dimethylbenzidine, and 3,3'-dimethoxybenzidine are used primarily in the production of dyes and pigments for the coloring of textiles, paper, leather, rubber,

plastics, inks, paints, lacquers, and enamels. Other less extensive uses of these compounds are as reagents in the detection of inorganic ions, organic molecules, and occult blood, as stains in microscopy, and as a curing agent for polyurethane elastomers. 4-Aminobiphenyl has been used as a rubber antioxidant.

6. Data are not available concerning the distribution of benzidine compounds in the atmosphere or soil.

7. Benzidine and its derivatives are thought to present a hazard in water only near factories which use or manufacture these compounds. No data exist on benzidine in drinking water.

8. No data are available on accumulation in and movement through food chains, except for a recent radiometric study indicating that bluegill fish can bioconcentrate benzidine to a 44-fold increase over concentrations in water.

9. Toxic effects to plants and to wild and domestic animals are not known except for recent laboratory studies on a very limited number of species.

10. The principal avenues of benzidine absorption by humans are dermal and pulmonary.

11. Benzidine does not appear to accumulate in human body tissues, but the evidence is indirect and is based on measurements of urinary concentrations in exposed workers.

12. Benzidine compounds do not accumulate in tissues of the usual laboratory animals, although unequal concentrations appear in different organs following administration. Concentrations tend to be high in those organs prone to later tumor development.

13. Although there is no direct evidence of the placental transfer of these compounds, studies in mice and rats point to this possibility.

14. Benzidine compounds are believed to become active carcinogens only after metabolic transformations that involve N-hydroxylation to produce the proximate carcinogens, followed by esterification and possibly conjugation with sulfate or glucuronate to produce the ultimate carcinogens.

15. Benzidine compounds and metabolites are eliminated from the animal body via the urine, bile, and feces.

16. Benzidine compounds cause dermatitis, cystitis, and hematuria in humans.

17. Benzidine is believed to be carcinogenic in humans and a major cause of bladder cancer among exposed industrial workers.

18. A recent report also implicated 3,3'-dichlorobenzidine as a cause of certain types of cancer among exposed industrial workers, but there are no reports of its association with bladder cancer.

19. Although there are no reports of any associations between cancer in humans and exposure to 3,3'-dimethylbenzidine or 3,3'-dimethoxybenzidine, these compounds induce a variety of cancers in experimental animals and must therefore be considered as potentially hazardous to humans.

20. Although 4-aminobiphenyl is believed to cause bladder cancer in humans, its manufacture and use in the United States today are considered to be very low.

21. Areas of research needed include: (1) epidemiological studies designed to determine relative risks and incidence rates of bladder cancer and other forms of cancer among adequate numbers of exposed and nonexposed workers, with careful consideration of confounding factors such as age, smoking history, and exposure to other potentially hazardous chemicals; (2) plant monitoring studies to better establish the degree of skin and pulmonary involvement in absorption; (3) pharmacokinetic studies to better substantiate mechanisms and extent of body transport and storage; (4) more extensive monitoring and surveillance of users of these compounds outside of large industrial plants, including those who use aerosol formulations of paints, lacquers, or enamels containing benzidine yellow; (5) environmental monitoring studies involving attempts to measure these compounds in air, water, sediment, soil, and selected species of aquatic and terrestrial life; and (6) laboratory studies aimed at determining the extent of toxic effects and possible bioaccumulation in a variety of aquatic and terrestrial species.

SECTION 2

PHYSICAL AND CHEMICAL PROPERTIES AND ANALYSIS

2.1 SUMMARY

Benzidine is a synthetic aromatic diamine not known to occur naturally. It was first synthesized in 1845 and has since become a versatile and important chemical coupling agent in the manufacture of commercial azo dyes. These dyes form the largest and most useful class of all commercial dyes; many of them are of great economic importance in the textile industry because of their ability to dye cotton directly without the use of mordants. Benzidine is also widely used in analytical chemistry and biological laboratories where its versatile chemistry makes it a useful reagent in the detection and determination of many inorganic ions as well as hydrogen peroxide, nicotine, sugars, occult blood, and bacterial cytochromes. Benzidine is sometimes used in security printing because it reacts with ink erasers to give colored products which reveal attempted alterations. About 680,400 kg of benzidine was produced in the United States in 1972.

Despite its versatile chemistry and widespread use, benzidine is a hazardous substance which is believed to be a potent bladder carcinogen. Apparently, a metabolite, rather than the aromatic amine per se, is the ultimate carcinogen. Most investigators have therefore been concerned with identifying and testing the carcinogenicity of the metabolic products of aromatic amines in various species. Results from early studies gave rise in 1952 to the hypothesis that carcinogenicity is due to the formation of *ortho*-hydroxy metabolites of aromatic amines. Later results suggested the generally held current theory that formation of an N-hydroxy metabolite is the first step in the activation of aromatic amines and amides for carcinogenesis. According to this view, subsequent esterification of the N-hydroxylated metabolite produces a highly reactive electrophile, the ultimate carcinogen, that attacks nucleophilic tissue components such as DNA and RNA. Experimental work verifying this chemical sequence in the metabolism of benzidine has not been performed, but firm evidence exists for the presence of an acetyl-N-hydroxy metabolite in the urine of persons exposed to benzidine.

Several benzidine derivatives are also used extensively in the United States. 3,3'-Dichlorobenzidine is employed as a coupling agent in the manufacture of dyes and pigments which color plastic resins, rubber, printing inks, metal finishes, textiles, and wallpaper prints. During 1971, about 1.6 million kg of this compound was manufactured in the United States and an additional 635,000 kg was imported. Other widely used derivatives of benzidine include 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine. These compounds are components of nearly 100 different dyes listed in the *Colour Index*. More than 44,000 kg of 3,3'-dimethylbenzidine and 122,000 kg of 3,3'-dimethoxybenzidine were imported into the United States during 1970 and 1971 respectively. Only limited information about the metabolic chemistry of these compounds is available. Other pertinent congeners of benzidine include the nitro, alkoxy, sulfonic acid, and carboxylic acid derivatives as well as 4-aminobiphenyl and 4-nitrobiphenyl. Few metabolic or carcinogenic studies have been performed with these benzidine derivatives. 4-Aminobiphenyl is an established carcinogen whose large-scale commercial production in the United States apparently ceased in 1955 when its carcinogenic character was generally recognized. 4-Nitrobiphenyl is readily reduced in vitro and in vivo to 4-aminobiphenyl; therefore, it is as hazardous as 4-aminobiphenyl. 4-Nitrobiphenyl was used chiefly as an intermediate in the synthesis of 4-aminobiphenyl. It apparently has not been produced in large quantities in the United States since the use of 4-aminobiphenyl was discontinued; however, small amounts of both 4-aminobiphenyl and 4-nitrobiphenyl are still manufactured for research and pilot plant operations.

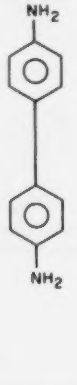
Approved analytical methods for determining benzidine were first established by the Association of British Chemical Manufacturers in 1954. These colorimetric methods were used with occasional innovations for almost all of the quantitative analyses of benzidine which were subsequently reported. Recently, federal legislation in this country provided an impetus for the development of more sensitive and precise methods for quantifying benzidine and its congeners; the end results of this stimulation are not yet apparent. Gas chromatographic and spectrophotofluorometric methods appear to be attractive alternatives to the older colorimetric procedures, but further experience will be needed to fully evaluate the new methods. Meanwhile, until an adequately tested method is officially sanctioned, the U.S. Environmental Protection Agency has designated a recently revised version of the older chloramine-T colorimetric technique as the interim method of determining benzidine.

2.2 PHYSICAL AND CHEMICAL PROPERTIES

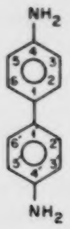
In January 1974, the U.S. Department of Labor issued occupational safety and health standards for 14 chemical compounds known to cause, or suspected of causing, human cancer from occupational exposure (*Federal Register*, 1974). Benzidine and 3,3'-dichlorobenzidine were included among these compounds. Pertinent physical and chemical properties of benzidine and relevant congeners are discussed in the following sections; established biotransformation products and urinary metabolites are also characterized.

2.2.1 Benzidine and Salts

First prepared by Zinin in 1845 (Stecher, 1968), benzidine is now a versatile and important intermediate in the synthesis of commercial dyes (International Agency for Research on Cancer, 1972, p. 80). Its composition and structure are indicated by the formulas $C_{12}H_{12}N_2$ and

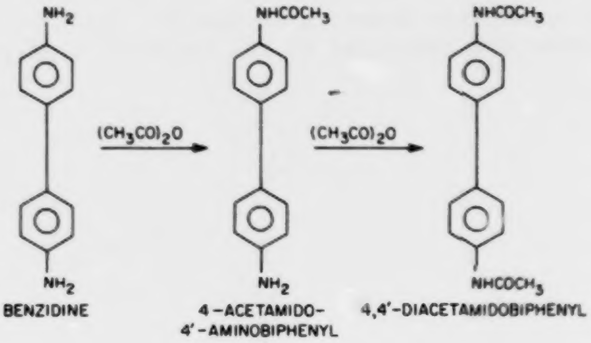


the *Chemical Abstracts* identification number of benzidine is 92875; it is also known as biphenyl, 4,4'-diamino-, 4,4'-biphenyldiamine, 4,4'-diaminobiphenyl, 4,4'-diphenylenediamine, 4,4'-diaminodiphenyl, *p*-diaminodiphenyl, *Colour Index* (CI) azoic diazo component 112, benzidine base, and Fast Corinth Base B (Christensen and Luginbyhl, 1974). The numbering system commonly used in describing benzidine and its derivatives is indicated in the following diagram:

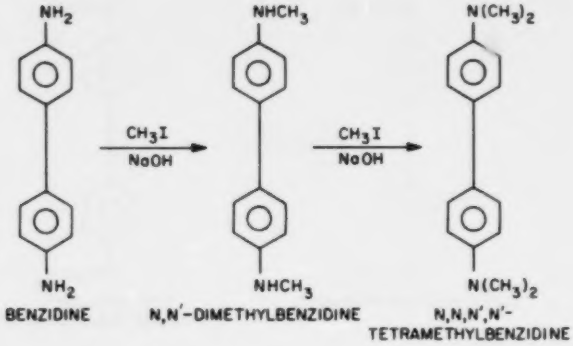


2.2.1.1 Physical Properties — Benzidine is a colorless, crystalline compound which has a molecular weight of 184.23, a density of 1.250 (20/4°C), a melting point of 115°C to 120°C (slow heating) or 128°C (rapid heating), and a boiling point of about 400°C at 740 torr (Stecher, 1968). It is soluble in both alcohol and ether but only slightly soluble in water (0.52 mg/ml at 25°C) (Bowman, King, and Holder, 1976). Benzidine forms colorless, crystalline salts with hydrochloric acid ($C_{12}H_{12}N_2 \cdot 2HCl$) and sulfuric acid ($C_{12}H_{12}N_2 \cdot H_2SO_4$); the former is soluble in water (61.7 mg/ml at 25°C) (Bowman, King, and Holder, 1976) and alcohol, but the latter is only slightly soluble in those solvents.

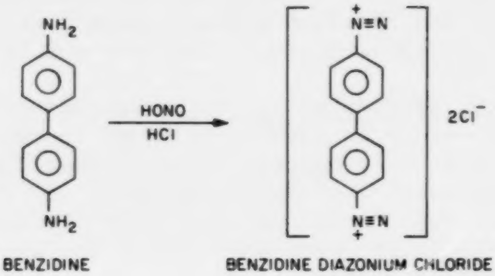
2.2.1.2 Chemical Properties — Benzidine is both a primary aromatic amine and a diphenyl derivative; it undergoes chemical reactions characteristic of each of these classes of compounds as well as some which are peculiar to it alone. Although weakly basic ($pK_b = 12.13$), benzidine forms stable salts with mineral acids (Karrer, 1950, p. 453); it also forms complexes with a large number of metallic salts (Welcher, 1947, p. 277). Like other primary and secondary amines, benzidine is sensitive to oxidation, and pure colorless crystals darken with decomposition products during storage unless protected from atmospheric oxygen (Fieser and Fieser, 1956, p. 604). Benzidine is easily acetylated,



alkylated,

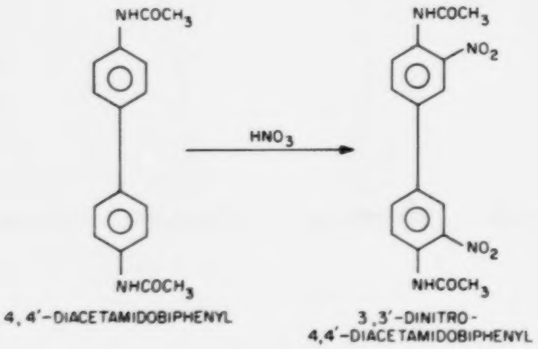


and diazotized (International Agency for Research on Cancer, 1972, p. 80),

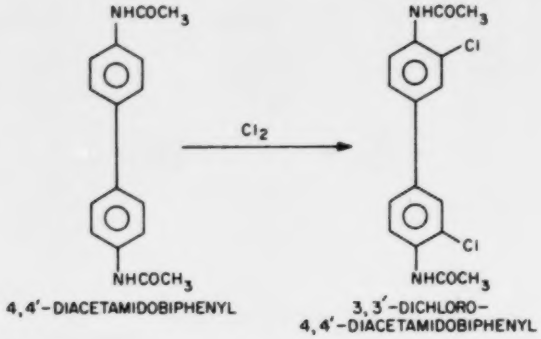


The latter compound, tetrazotized benzidine, couples with many different aromatic amines and phenols to yield a wide variety of commercial dyes which can be used to color cotton fabrics without the use of mordants.

When the amino groups of benzidine are protected by acetylation, benzidine is also easily nitrated,



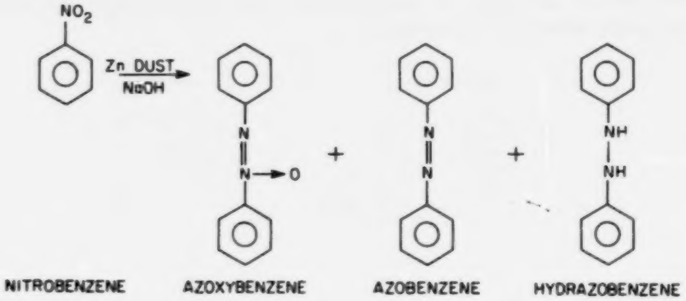
and halogenated,



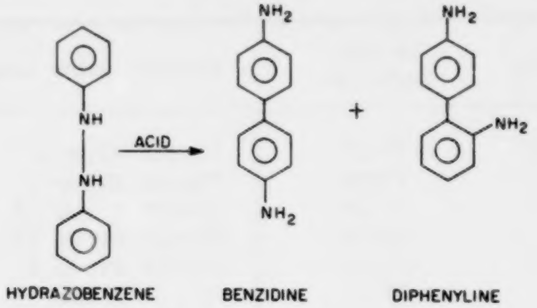
On treatment with sulfuric acid, benzidine forms a variety of sulfonic acids, benzidine sulfone, and sulfonic acids of the sulfone, depending on the concentration and amount of acid and the reaction temperature. Other benzidine reactions and reaction products have been reported in the literature (Lurie, 1964, p. 408); however, they are mainly of academic interest.

2.2.1.3 Occurrence, Synthesis, and Use — Benzidine is a synthetic substance not known to occur in nature, although it can occur in waste streams of facilities where it is produced or used. Benzidine can also be formed in situ by the introduction of benzidine-based azo dyes into waste streams containing hydrogen sulfide or sulfur dioxide (Takemura, Akiyama, and Nakajima, 1965); the latter compounds reduce the azo linkage of the dye, liberating the aromatic amine.

Benzidine is manufactured on a commercial scale by treating an alkaline solution of nitrobenzene with a mild reducing agent — usually zinc dust. The resulting solution contains a mixture of stepwise reduction products, azoxybenzene and azobenzene, but it consists primarily of the intermediate product, hydrazobenzene:



The addition of mineral acids to the hydrazobenzene solution causes spontaneous rearrangement of this compound into the desired product, benzidine:



This reaction is the well-known benzidine rearrangement (Karrer, 1950, p. 498). A small amount, usually 3% to 15%, of the *o*,*p*'-diaminobiphenyl (diphenyline) isomer is usually formed. Nitrobenzene also can be reduced with zinc amalgam, iron powder, noble metal or nickel catalysts, or electrolytically (Lurie, 1964, p. 411).

The principal use of benzidine and its salts is in the manufacture of dyestuffs based on the coupling of tetrazotized benzidine (Section 2.2.1.2) with phenols and aromatic amines. More than 250 commercial dyes based on benzidine have been reported (*Colour Index*, 1956); the most important of these are listed in Table 2.1. The composition and color of several important benzidine-based cotton dyes are listed in Table 2.2. The symbol → in Table 2.2 stands for "diazotized and coupled with" and is a shorthand designation commonly used by dye chemists. The chemical structure of Congo Red, one of the more important dyes in Table 2.2, is shown below (Johnson, Zenhäuser, and Zollinger, 1963):

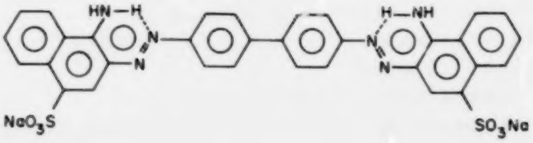


TABLE 2.1. COMMERCIAL DYES DERIVED FROM BENZIDINE

Colour Index name	*Colour Index* No.	*Colour Index* name	*Colour Index* No.
Mordant Yellow 36	14135	Direct Blue 6	22610
Pigment Red 39	21080	Direct Brown 1	30045
Direct Red 28	22120	Direct Brown 1A	30110
Direct Orange 8	22130	Direct Brown 154	30120
Direct Red 10	22145	Direct Brown 6	30140
Direct Red 13	22155	Direct Brown 95	30145
Direct Red 37	22240	Direct Black 38	30235
Direct Red 1	22310	Direct Black 4	30245
Direct Brown 2	22311	Direct Green 1	30280
Direct Orange 1	22370	Direct Green 6	30295
	22375	Direct Green 8	30315
	22430	Direct Brown 31	35660
Direct Violet 1	22570	Direct Brown 74	36300
Direct Blue 2	22590		

Source: Adapted from U.S. Tariff Commission, 1963, cited in Lurie, 1964, Table 1, p. 413. Reprinted by permission of the publisher.

TABLE 2.2. COTTON DYES DERIVED FROM BENZIDINE

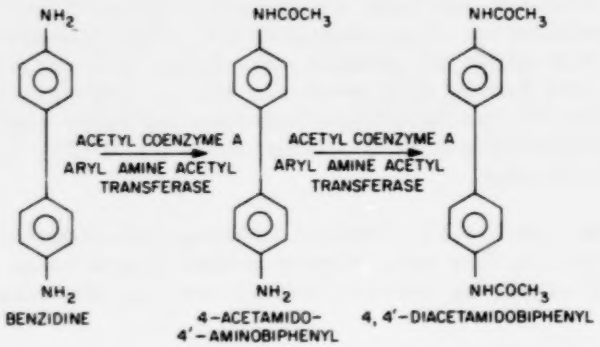
Composition	Color
Salicylic acid ← benzidine → naphthionic acid	Orange
Naphthionic acid ← benzidine → naphthionic acid	Congo Red
H acid ← benzidine → H acid	Blue
Salicylic acid ← benzidine → gamma acid	Brown
Salicylic acid ← benzidine → H acid ← *p*-nitroaniline	Green
m-Phenylenediamine ↔ benzidine → H acid ← aniline	Black

Source: Adapted from Lurie, 1964, Table 2, p. 414. Reprinted by permission of the publisher.

Although the manufacture of dyestuffs accounts for the major portion of the benzidine consumed in the United States, there are many other uses for this compound. Benzidine is employed in analytical chemistry to detect and determine inorganic ions such as cadmium, copper, manganese, chloride, fluoride, cyanide, ferricyanide, ferrocyanide, phosphate, silica, sulfate, tungstate, hypohalites, permanganate, nitrate, nitrite, and phosphomolybdate (Welcher, 1947). Benzidine is used as a chromogenic spray reagent in the thin-layer chromatography of chlorinated organic pesticides (Adamovic, 1966). It is used as a reagent for determining hydrogen peroxide, nicotine, sugars, occult blood, and bacterial cytochromes. Benzidine is sometimes used in security printing because it reacts with ink erasers to give colored products (International Agency for Research on Cancer, 1972, p. 81).

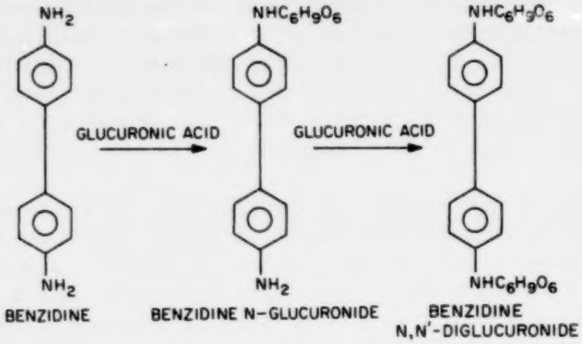
2.2.1.4 Biochemical Reactions and Metabolites — The long-held suspicion that benzidine is a bladder carcinogen (Hueper and Conway, 1964, p. 268) was established statistically by Case et al. (1954) and was subsequently verified by experiments with rats, dogs, hamsters, and mice (*Federal Register*, 1974). Benzidine is also regarded as mutagenic (Ames et al., 1973). The specific biochemical reactions responsible for these physiological effects are not yet known (Miller and Miller, 1971*a*, p. 84); some doubt even remains about the identity of certain metabolites (Haley, 1975). Nevertheless, an appreciable store of metabolic chemistry exists, and the patterns of biotransformations for aromatic amines pointed out by Williams (1959, p. 428) probably are generally applicable to benzidine. These characteristic reactions will be used as the basis of the biochemistry discussed below. However, the reader is cautioned that most reactions described in this section remain largely unsubstantiated in the literature since very little work has been done to characterize biochemical reactions of benzidine.

Unlike their aliphatic analogues, aromatic amines are not deaminized in vivo; instead, the amino group is conjugated and the aromatic ring is hydroxylated. Conjugates of aromatic amines are more polar and less lipid soluble than their precursors and consequently are more readily excreted in the urine (Parke, 1968, p. 4). When a molecule, such as benzidine, has more than one functional group, only one group is usually conjugated. Double conjugates are formed in multifunctional substrates only if conjugation of a single functional group does not increase the polarity of the molecule sufficiently to promote rapid excretion. The most common conjugation of the amino group is acetylation (Section 2.2.1.2), a reaction in which the amino group is converted to an acetamido group:

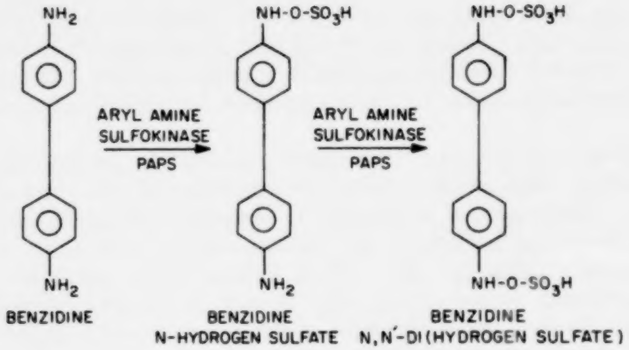

NH2
NH2
BENZIDINE
ACETYL COENZYME A
ARYL AMINE ACETYL TRANSFERASE
NHCOCH3
NH2
4-ACETAMIDO-4'-AMINOBIPHENYL
ACETYL COENZYME A
ARYL AMINE ACETYL TRANSFERASE
NHCOCH3
NHCOCH3
4, 4'-DIACETAMIDOBIPHENYL

Acetylation of benzidine is thought to require the cofactor acetyl coenzyme A as well as an enzyme, aryl amine acetyl transferase.

The aromatic amino group also conjugates with glucuronic and sulfuric acids to form N-glucuronides,

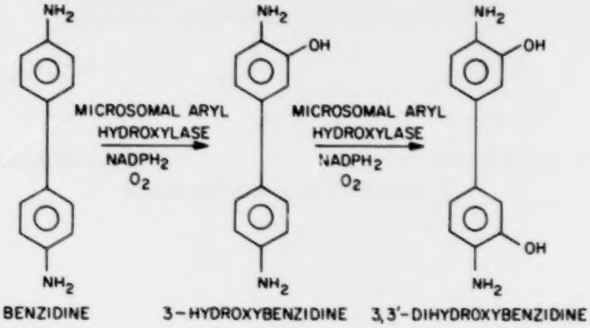


and N-hydrogen sulfates,



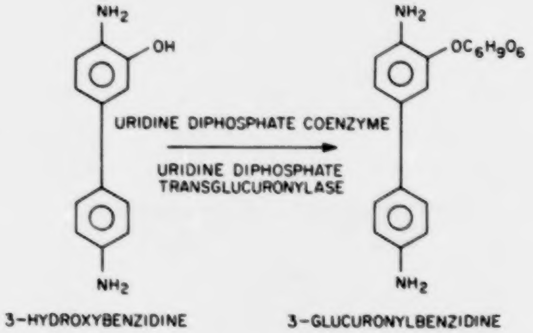
Conjugation with glucuronic acid, the most important conjugation mechanism, occurs in all mammals and most vertebrates except fish (Parke, 1968, p. 77). Aryl amine glucuronides form spontaneously from glucuronic acid and aryl amine, but glucuronides of phenols, alcohols, and carboxylic acids (O-glucuronides) are formed only with the aid of appropriate enzymes. Similarly, formation of the benzidine hydrogen sulfates requires the adenosine coenzyme 3'-phosphoadenosine-5'-phosphosulfate (PAPS) and the appropriate aryl amine sulfokinase.

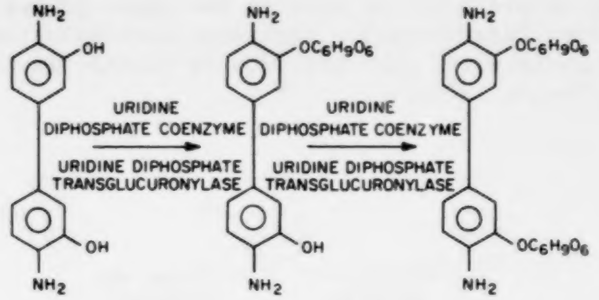
The second type of biochemical behavior of aromatic amines, hydroxylation of the ring, occurs extensively unless there is an alternative metabolic reaction involving another substituent in the ring. Usually, the hydroxyl group enters *ortho* or *para* to the amino groups, depending on the presence of other substituents. Benzidine hydroxylation occurs *ortho* to the amino group since the *para* position is blocked in the parent compound (Sciarini and Meigs, 1961*a*):



A hepatic microsomal enzyme, oxygen, and reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$) are required for the hydroxylation reaction. At least two distinct mechanisms can operate; the above reaction appears to account for the reported metabolites of benzidine (Haley, 1975; Parke, 1968, p. 40).

The hydroxybenzidines shown in the previous reactions may be excreted unchanged; however, they are normally conjugated with glucuronic acid,

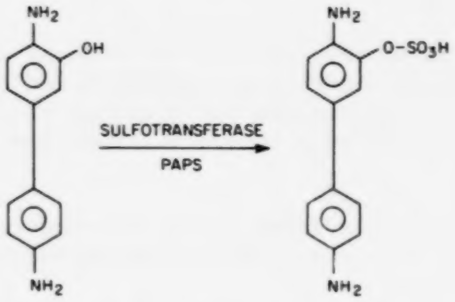

NH2
OH
OC6H9O6
URIDINE DIPHOSPHATE COENZYME
URIDINE DIPHOSPHATE TRANSGLUCURONYLASE
3-HYDROXYBENZIDINE
3-GLUCURONYLBENZIDINE



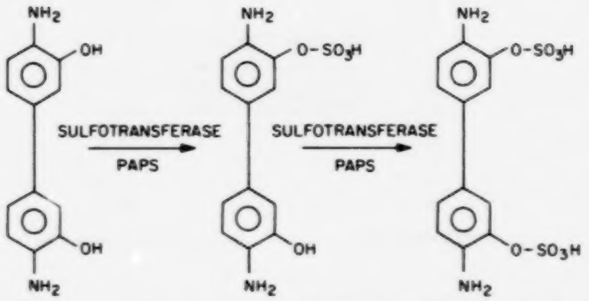
3,3′-DIHYDROXYBENZIDINE 3,3′-DIGLUCURONYLBENZIDINE

3-HYDROXY-3′-GLUCURONYLBENZIDINE

or sulfuric acid,



3-HYDROXYBENZIDINE BENZIDINE 3-HYDROGEN SULFATE

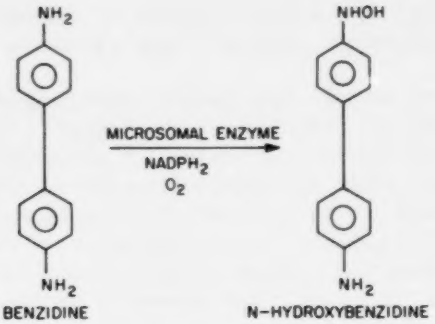


3,3′-DIHYDROXYBENZIDINE BENZIDINE 3,3′-DI(HYDROGEN SULFATE)

BENZIDINE 3-HYDROXY-3′-HYDROGEN SULFATE

Conjugation of the hydroxyl groups of hydroxybenzidine with glucuronic acid is not spontaneous as was the previously described reaction of the amino groups in benzidine. Uridine diphosphate coenzyme and uridine diphosphate transglucuronylase are required. An enzyme is also required for formation of the sulfate esters. These compounds and the N-hydrogen sulfates mentioned previously are often identified in the literature as etheral sulfates because of their solubility in, and easy extraction into, ether solvents.

In 1960, an in vivo biochemical reaction was discovered in which aromatic amines are oxidized to hydroxylamines (Cramer, Miller, and Miller, 1960). This process, called N-hydroxylation, occurs widely (Hathway, 1970, p. 271) and is now considered by some investigators to be the first step in the activation of aromatic amines and amides for carcinogenesis (Miller and Miller, 1971*a*):



The N-hydroxylating system requires a microsomal enzyme, $NADPH_2$, and oxygen; the necessary enzyme occurs in the liver, lungs, and bladder (Parke, 1968, p. 47).

Another type of metabolic process, different from those discussed above, is the in vivo chemical reaction by which benzidine is produced from a previously ingested source material such as an azo dye. It has long been known that humans and other mammals can metabolically reduce certain dyes to free carcinogenic aromatic amines (Fuller, 1937; Williams, 1949, pp. 150-154). Rinde and Troll (1975) established that metabolic reduction of benzidine-based azo dyes can occur in the monkey. They found substantial amounts of benzidine and monoacetylbenzidine in the urine of animals which were fed single doses of benzidine-based dyes (Direct Red 28, Direct Blue 6, Direct Black 38, and Direct Brown 95) dissolved in dimethyl sulfoxide. In view of their findings, these investigators recommended restricting the production of azo dyes to those which could be manufactured from noncarcinogenic aromatic amines, such as aniline. Discussions of metabolic transformations in general and of aromatic amines in particular may be found in Arrhenius (1974), Hathway (1970), Parke (1968), and Walker (1970).

The simple metabolic products indicated in the various biochemical reactions described above are not necessarily the compounds excreted when benzidine is ingested by the host; successive operations on a metabolite by different biochemical processes may result in a complex end product. Furthermore, use of a particular biotransformation reaction varies among animal species and with experimental conditions. It is necessary, therefore, to identify the metabolites of each animal species experimentally.

2.2.1.5 **Chemical Structure Associated with Carcinogenic Activity of Aromatic Amines** — Chemicals were first used to produce experimental tumors more than 50 years ago (Yamagiwa and Ichikawa, 1918). Since then, carcinogenic activity has been demonstrated for a wide variety of chemical compounds (Clayson, 1962; Hueper and Conway, 1964; International Agency for Research on Cancer, 1972, 1973*a*, 1973*b*, 1974*a*, 1974*b*). These compounds are structurally diverse and have no common metabolic patterns (Miller and Miller, 1966); no general explanation for their carcinogenic activity is available. Some advances have been made, however, in understanding the carcinogenic activity of certain classes of compounds. The following discussion deals with aromatic amines — the class to which benzidine belongs.

Not all aromatic amines are potent carcinogens; to qualify, the amino group apparently must be attached to an aromatic system containing more than one ring. Furthermore, the point of attachment of the amino group to the ring must be such that the position *para* to the amino group is occupied by another substituent or by part of the conjugated system (Clayson, 1962, p. 211). Unlike carcinogenic hydrocarbons, carcinogenic aromatic amines generally induce tumors at tissue sites distant from the point of administration. This behavior suggests that aromatic amines are not carcinogenic per se but acquire this characteristic only after metabolic activation.

Since hydroxylation of the ring occurs extensively in these compounds (Section 2.2.1.4), hydroxylated derivatives were among the first metabolites suspected as carcinogens (Hueper, 1938). In addition, since the amino group is an *ortho-para* director and since the *para* position in carcinogenic aromatic amines is usually blocked, hydroxylation of these compounds characteristically occurs in the *ortho* position. Early workers thus developed the *ortho*-hydroxylation hypothesis (Clayson, 1962, p. 219) as a guide to identifying potentially carcinogenic aromatic amines. Acceptance of the theory diminished, however, when some *o*-hydroxy amines failed to produce tumors and others failed to produce neoplasms when administered by a different route (Miller and Miller, 1966).

Although strong evidence exists for the carcinogenicity in mice of *o*-hydroxyanthranilic acid (Ehrhart, Georgii, and Stanislawski, 1959) and some *o*-hydroxyamine metabolites of tryptophan (Bryan, Brown, and Price, 1964), the *ortho*-hydroxylation hypothesis was largely displaced in 1960 by the discovery of the in vivo reaction of N-hydroxylation (Section 2.2.1.4) in which the nitrogen of aromatic amines is oxidized enzymatically to hydroxylamine (Cramer, Miller, and Miller, 1960). Troll, Belman, and Rinde (1963) established that this metabolite of benzidine occurs in humans. N-Hydroxylation of aromatic amines and amides occurs widely and N-hydroxy metabolites of carcinogenic aromatic amines are at least as active as the parent carcinogen (Boyland, Dukes, and Grover, 1963; Miller, Enomoto, and Miller, 1962; Miller, Miller, and Enomoto, 1964; Poirier et al., 1965). Also, unlike the *o*-hydroxy compounds, the N-hydroxy metabolites can induce tumors at sites where the parent compounds are inactive (Miller and Miller, 1966). As a result of these studies, N-hydroxylation is now widely recognized as the first step in the activation of aromatic amines and amides for carcinogenesis (Miller and Miller, 1971*a*).

Although carcinogenic, N-hydroxy aromatic amines lack the electrophilic properties of ultimate carcinogens which enable the latter to bind to nucleophilic sites of proteins and nucleic acids at physiological pH (Miller, 1970). This deficiency indicates that N-hydroxy aromatic amines are proximate rather than ultimate carcinogens. The chemical reactivity of esters of aromatic hydroxylamines and hydroxamic acids suggests that esterification (Section 2.2.1.4) of N-hydroxylated metabolites is the next step in the metabolic sequence leading to the ultimate carcinogen (Miller and Miller, 1972). No experimental work establishing this chemical sequence for the metabolism of benzidine has been performed; however, such experimental evidence has been obtained for other aromatic amines, namely, 2-acetylaminofluorene (AAF) and N-methyl-4-aminoazobenzene (MAB). Miller and Miller (1972) reported that after treating male rats with AAF, the major reactive metabolites in the liver were sulfuric acid esters which, when desulfatized, yielded an aminonium cation that reacted readily with nucleophilic tissue components such as proteins, RNA, and DNA (Miller and Miller, 1971*a*). The principal steps leading to the formation of the reactive sulfates and the aminonium cations of AAF and MAB are shown in Figure 2.1.

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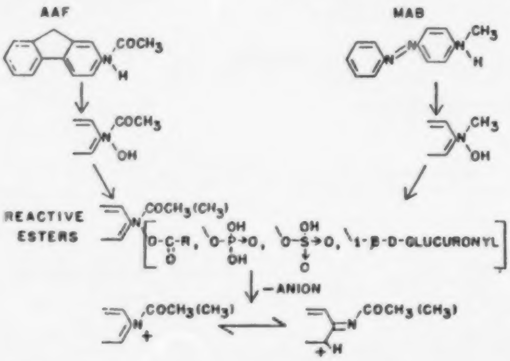

Figure 2.1. Formation of reactive esters and aminonium cations from 2-acetylaminofluorene (AAF) and N-methyl-4-aminoazobenzene (MAB). Source: Miller and Miller, 1969*a*, Figure 5, p. 297. Reprinted by permission of S. Karger AG, Basel.

A variety of amino acid residues in proteins and bases in nucleic acids are attacked by electrophilic reactants derived from aromatic amine carcinogens. Among nucleic acids and proteins, the principal targets appear to be guanine, phosphate, methionine, cysteine, tyrosine, and histidine (Miller and Miller, 1969*b*). The manner in which two of these, methionine and (deoxy)guanosine, react in vitro with N-acetoxy-AAF is shown in Figures 2.2 and 2.3. In each instance, identical metabolites are also obtained from the liver proteins or hepatic RNA of rats fed AAF. As shown in Figure 2.2, the methionine adducts of N-acetoxy-AAF decompose readily to form 1- and 3-methylmercapto-AAF, a process which can result in cleavage of methionyl peptide bonds and in modification of the reacted protein (Miller and Miller, 1969*b*). Such reactions could have significant consequences in terms of enzymatic activity of alterations in substrate specificity. The reaction of N-esters of AAF with guanine residues, the major site of attack in nucleic acids (Miller and Miller, 1971*b*), has obvious implications relative to both genetic and epigenetic mechanisms of chemical carcinogenesis. The alteration of guanine bases in DNA by insertion of bulky AAF groups in the 8-position could impede the progress of this enzyme along the double helix and lead to deletion mutants. Indeed, deletion mutants are the principal type observed when *Drosophila* is treated with N-acetoxy-AAF (Fahmy and Fahmy, 1967). However, single base pair changes occur predominantly when *Bacillus subtilis* is treated with the same mutagen (Miller and Miller, 1969*b*).

From the foregoing discussion it is apparent that considerable progress has been made in recent years in identifying proximate and ultimate aromatic amine carcinogens and the mechanisms by which they are formed.

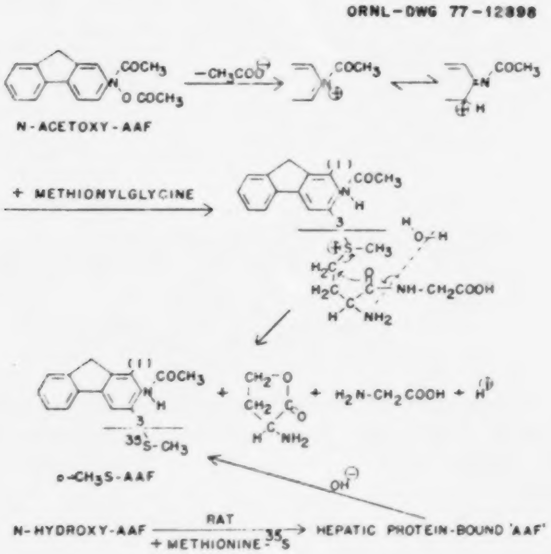

Figure 2.2. The in vitro reaction of N-acetoxy-AAF and methionylglycine. Source: Miller and Miller, 1969*b*, Figure 3, p. 244. Reprinted by permission of the publisher.

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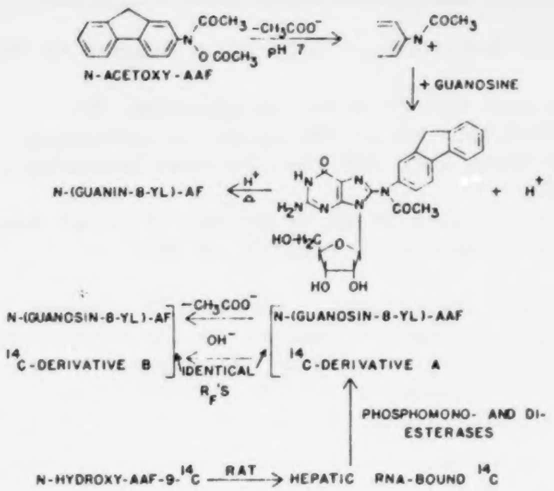


Figure 2.3. The in vivo reaction of N-acetoxy-AAF with guanosine. Source: Miller and Miller, 1969*b*, Figure 4, p. 244. Reprinted by permission of the publisher.

Evidence is also accumulating relative to the nucleophilic targets of these compounds; however, there is no definitive information concerning the cellular chemistry of the carcinogenic process itself — no understanding of the carcinogenic mechanism in molecular terms. Future work must address this difficult aspect of the problem. Commonly postulated mechanisms of chemical carcinogenesis are outlined in Table 2.3. Chemical structures associated with the carcinogenic activity of aromatic amines are discussed further by Clayson (1975), Conney and Levin (1974), and Heidelberger (1970).

2.2.2 3,3'-Dichlorobenzidine and Salts

3,3'-Dichlorobenzidine is a widely used chemical intermediate which has been produced commercially for more than 35 years (U.S. Tariff Commission, 1938, cited in International Agency for Research on Cancer, 1974*a*). Its composition and structure are indicated by the formulas $C_{12}H_{10}Cl_2N_2$ and

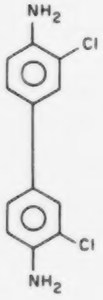


TABLE 2.3. POSSIBLE MECHANISMS OF CHEMICAL CARCINOGENESIS

I. Genetic mechanisms — heritable changes in DNA genome via:
  1. Direct modification of existing DNA
  2. Modification of RNA which is subsequently transcribed into DNA that becomes integrated into host DNA
  3. Alterations which decrease, at least temporarily, the fidelity of copying of DNA

II. Epigenetic mechanisms — nongenomic changes leading to:
  1. Quasi-permanent changes in the transcription of DNA (including integrated virus genomes and oncogenes)
  2. The preferential proliferation of previously existing preneoplastic or neoplastic cells

Source: Miller and Miller, 1971*b*, Table 1, p. x.

The *Chemical Abstracts* identification number of 3,3'-dichlorobenzidine is 91-94-1. It is also known as CI 23060, DCB, 4,4'-diamino-3,3'-dichlorobiphenyl, *o*,*o*'-dichlorobenzidine, dichlorobenzidine, dichlorobenzidine base, 3,3'-dichloro-4,4'-biphenyldiamine, and 3,3'-dichlorobiphenyl-4,4'-diamine.

2.2.2.1 Physical and Chemical Properties — 3,3'-Dichlorobenzidine is a colorless, crystalline compound which has a molecular weight of 253.1 and a melting point of 132°C to 133°C. It is nearly insoluble in cold water, slightly soluble in dilute hydrochloric acid, and readily soluble in ethanol, benzene, and glacial acetic acid (International Agency for Research on Cancer, 1974*a*). 3,3'-Dichlorobenzidine forms salts with hydrochloric acid. The principal physical properties of the dihydrochloride salt are shown in Table 2.4.

The chemistry of 3,3'-dichlorobenzidine is similar to that of aromatic primary amines in general and benzidine in particular (Section 2.2.1.2). Its most important chemical reaction is the formation of the tetrazonium compound which couples with aromatic amines and phenols to form a variety of dyes and pigments. In general, the chlorine atoms of 3,3'-dichlorobenzidine are passive and do not participate in these reactions (Gerarde and Gerarde, 1974; Karrer, 1950, p. 414).

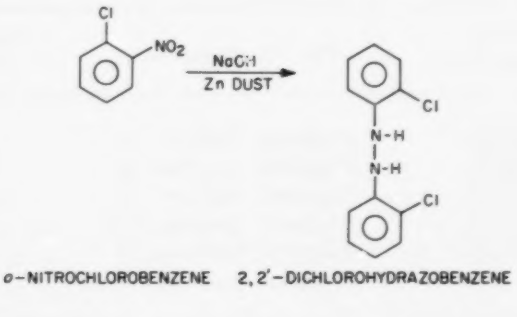
2.2.2.2 Occurrence, Synthesis, and Use — 3,3'Dichlorobenzidine is not known to occur naturally, although it may be present in the waste streams from production facilities where it is manufactured or used (International Agency for Research on Cancer, 1974*a*). There is also evidence that 3,3'-dichlorobenzidine is regenerated metabolically in rabbits and humans from an organic pigment (benzidine yellow) in which it is used as a coupling agent (Akiyama, 1970).

TABLE 2.4. PHYSICAL PROPERTIES OF
3,3'-DICHLOROBENZIDINE DIHYDROCHLORIDE

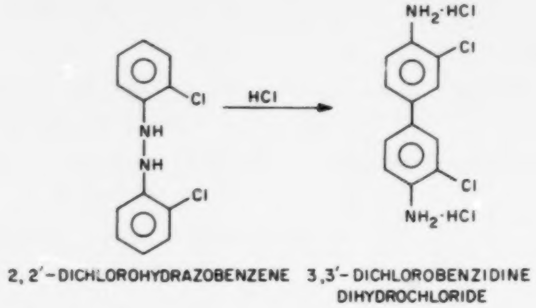
Formula	$C_{12}H_{10}Cl_2N_2 \cdot 2HCl$
Molecular weight	326.1
Flash point (Cleveland open cup)	No flammable concentration of vapors evolved at 200°C
Auto and ignition temperature	No exothermic reaction recorded up to 300°C
Corrosiveness	Contains free mineral acidity or hydrochloric acid
Physical state	Light grayish semidry powder (approx 5% water); white to light gray semidry paste
Solubility	Slightly soluble in water and organic solvents; soluble in acids
Odor	Slightly pungent
Vapor pressure	Zero at room temperature
Particle size	Salt crystals; similar to table salt

Source: Adapted from Gerarde and Gerarde, 1974, Table 2, p. 324. Reprinted by permission of the publisher.

3,3'-Dichlorobenzidine is synthesized on a commercial scale by chemical reactions similar to those used for benzidine, except that the starting material is *o*-nitrochlorobenzene rather than nitrobenzene:



On treatment with sulfuric or hydrochloric acid, the intermediate 2,2'-dichlorohydrazobenzene undergoes the benzidine rearrangement (Karrer, 1950, p. 409) to form the 3,3'-dichlorobenzidine salt:


Cl
NH
NH
Cl
HCl
NH₂·HCl
Cl
Cl
NH₂·HCl
2,2'-DICHLOROHYDRAZOBENZENE
3,3'-DICHLOROBENZIDINE DIHYDROCHLORIDE

As with benzidine, the principal use of 3,3'-dichlorobenzidine is in the manufacture of dyestuffs based on the coupling of the tetrazotized compound with phenols and aromatic amines; 13 such dyes are described in the third edition of the *Colour Index* (1971). The 1971 production rates for six of these dyes are listed in Table 2.5. These dichlorobenzidine compounds are used chiefly as pigments in plastic resins, lacquer, rubber, printing inks, metal finishes, and textile and wallpaper prints (*Colour Index*, 1971) and in interior grade, lead-free finishes (paints), toy enamels, and floor coverings (Martens, 1968, p. 367).

Dichlorobenzidine is also used as a curing agent for liquid-castable polyurethane elastomers (Woolrich and Rye, 1969). However, compared to dye production, this use of dichlorobenzidine is relatively minor; it currently amounts to only a few hundred thousand kilograms per year in the United States (International Agency for Research on Cancer, 1974*a*).

TABLE 2.5. SOME DYES OR PIGMENTS MANUFACTURED FROM 3,3'-DICHLOROBENZIDINE IN 1971

Colour Index No.	Dye or pigment	Production (10^3 kg)
21090	Pigment Yellow 12	2547
21095	Pigment Yellow 14	1034
21105	Pigment Yellow 17	260
21110	Pigment Orange 13	65
21115	Pigment Orange 34	55
21120	Pigment Red 38	60

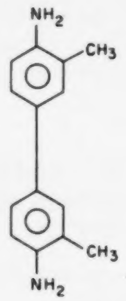
Source: Adapted from U.S. Tariff Commission, 1972, cited in International Agency for Research on Cancer, 1974*a*, p. 51.

2.2.2.3 Biochemical Reactions and Metabolites — On a purely chemical basis, the biochemistry of 3,3'-dichlorobenzidine could be expected to differ appreciably from that of its parent compound, benzidine. First, loss of halogens from the benzene ring apparently does not occur in vivo (Gerarde and Gerarde, 1974); thus, there is little probability that the chlorinated compound could form the 3-hydroxy derivatives characteristic of benzidine metabolites. Secondly, with the 1, 1', 3, and 3' positions in the chlorinated compound already blocked, the *ortho-para* directing influence of the amino groups should cause any additional substitutions to occur at the 5 or 5' positions. Such substitutions would result in metabolites appreciably different from those of benzidine.

The metabolism of 3,3'-dichlorobenzidine differs among species (Sciarini and Meigs, 1961*b*). In most investigations the excreted chlorinated compound appeared unchanged from its administered form. This apparent inertness of the chloro derivative may reflect the deactivating influence of halogen substituents on electrophilic aromatic substitution (Morrison and Boyd, 1974, p. 342).

2.2.3 3,3'-Dimethylbenzidine and Salts

3,3'-Dimethylbenzidine is a versatile, widely used dye intermediate; its elemental and structural composition is indicated by the formulas $C_{14}H_{16}N_2$ and



The *Chemical Abstracts* identification number of 3,3'-dimethylbenzidine is 119937. It is also known as diorthotolidine, 4,4'-diamino-3,3'-dimethylbiphenyl, 4,4'-diamino-3,3'-dimethyldiphenyl, 3,3'-dimethyl-4,4'-biphenyldiamine, 3,3'-dimethyl-4,4'-diphenyldiamine, 3,3'-dimethylbiphenyl-4,4'-diamine, 3,3'-dimethyldiphenyl-4,4'-diamine, 3,3'-tolidine, 4,4'-bi-*o*-toluidine, 4,4'-di-*o*-toluidine, diaminoditolyl, Fast Dark Blue Base R, and CI Azoic Diazo Component 113.

2.2.3.1 Physical and Chemical Properties — 3,3'Dimethylbenzidine is a white to reddish crystalline compound which has a molecular weight of 212.28 and a melting point of 131°C to 132°C. It is slightly soluble in water (1.3 mg/ml at 25°C) (Bowman, King, and Holder, 1976) and very soluble in ethanol, ethyl ether, and acetone (International Agency for Research on Cancer, 1972). 3,3'-Dimethylbenzidine is a weak base which readily forms salts with strong mineral acids such as hydrochloric and sulfuric. The monohydrochloride salt is soluble to the extent of 1 part in 112.4 parts of water at 12°C; the dihydrochloride, 1 part in 17.3 parts. The monosulfate salt is moderately soluble; the disulfate is difficultly soluble in water. The monohydrated, monacetyl derivative of 3,3'-dimethylbenzidine melts at 103°C; the diacetyl and tetraacetyl compounds at 315°C and 211°C respectively. 3,3'-Dimethylbenzidine forms an intense blue color with chlorine or bromine vapor (Lurie, 1964, p. 414). Like benzidine, its parent compound, it can also be hydroxylated, tetrazotized, and acetylated (Section 2.2.1.2).

2.2.3.2 Occurrence, Synthesis, and Use — 3,3'-Dimethylbenzidine is a chemical intermediate used in the preparation of more than 95 dyes listed in the *Colour Index* (International Agency for Research on Cancer, 1972, p. 88). Those manufactured in the United States during 1962 are listed in Table 2.6.

3,3'-Dimethylbenzidine is prepared in the manner described for benzidine (Section 2.2.1.3) except that *o*-nitrotoluene, rather than nitrobenzene, is the starting material (Lurie, 1964, p. 414). In addition to its use in the dye industry, 3,3'-dimethylbenzidine is also used extensively in qualitative and quantitative analyses for the detection and determination of cobalt, copper, gold, lead, manganese, mercury, silver, tungsten, iodide, nitrate, nitrite, and sulfate (Welcher, 1947, p. 462).

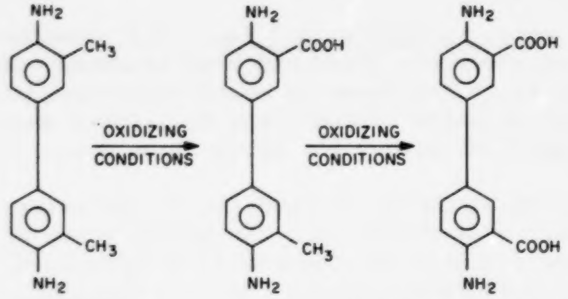
2.2.3.3 Biochemical Reactions and Metabolites — Little attention has been given thus far to the metabolic chemistry of 3,3'-dimethylbenzidine. In limited studies of dogs and humans, only the expected metabolic processes such as *ortho* hydroxylation, sulfation, and acetylation have been observed.

TABLE 2.6. COMMERCIAL DYES DERIVED FROM 3,3'-DIMETHYLBENZIDINE

Colour Index name	*Colour Index* No.
Direct Red 2	28500
Direct Red 39	23630
Acid Red 114	23635
Direct Blue 25	23790
Direct Blue 14	23850
Direct Blue 26	31930
Azoic Coupling Component 5	37610

Source: Adapted from U.S. Tariff Commission, 1963, cited in Lurie, 1964, Table 3, p. 415. Reprinted by permission of the publisher.

Since aliphatic side chains attached to a benzene ring are quite susceptible to oxidation in vitro (Morrison and Boyd, 1974, p. 384), Dieteren (1966) suggested that 3,3'-benzidinedicarboxylic acid may be a metabolic product of 3,3'-dimethylbenzidine:



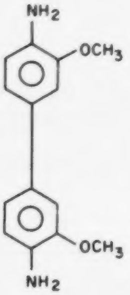
3,3'-DIMETHYLBENZIDINE 3,3'-BENZIDINEDICARBOXYLIC ACID

3-METHYL-3'-BENZIDINECARBOXYLIC ACID

However, no experimental evidence exists to support this suggestion.

2.2.4 3,3'-Dimethoxybenzidine and Salts

3,3'-Dimethoxybenzidine is a well-established commercial dye intermediate whose composition and structure are represented by the formulas $C_{14}H_{16}N_2O_2$ and



The *Chemical Abstracts* number of 3,3'-dimethoxybenzidine is 119-90-4. The compound is also known as bianisidine, 4,4'-diamino-3,3'-dimethoxybenzidine, di-*p*-amino-di-*m*-methoxydiphenyl, dianisidine, and 3,3'-dimethoxy-4,4'-diaminobiphenyl.

2.2.4.1 Physical and Chemical Properties — 3,3'-Dimethoxybenzidine is a colorless, crystalline compound which turns violet on standing and has a molecular weight of 244.3 and a melting point of 137°C to 138°C. The compound is poorly soluble in water (0.06 mg/ml at 25°C) (Bowman, King, and

Holder, 1976), but it is soluble in ethanol, ethyl ether, acetone, benzene, chloroform, and probably most organic solvents and lipids (International Agency for Research on Cancer, 1974*a*, p. 41). 3,3'-Dimethoxybenzidine is a weak base and forms salts with mineral acids; it has the chemical characteristics of primary aromatic amines in general and of benzidine in particular (Section 2.2.1.2).

2.2.4.2 Occurrence, Synthesis, and Use — 3,3'-Dimethoxybenzidine is an important dye intermediate which has been produced commercially for at least 50 years; it is not known to occur naturally, although it may sometimes be present in waste streams from facilities where it is manufactured or used (International Agency for Research on Cancer, 1974*a*, p. 43).

3,3'-Dimethoxybenzidine is prepared in the manner described for benzidine (Section 2.2.1.3) except that the methyl ether of *o*-nitrophenol is used as the starting material instead of nitrobenzene. Current U.S. production data on 3,3'-dimethoxybenzidine are unavailable. In 1967, five domestic producers manufactured 167,000 kg (U.S. Tariff Commission, 1968, cited in International Agency for Research on Cancer, 1974*a*). By 1971, only two domestic producers were reported; imports in that year totaled 124,000 kg (U.S. Tariff Commission, 1972, cited in International Agency for Research on Cancer, 1974*a*).

3,3'-Dimethoxybenzidine is the coupling agent in 89 dyes listed in the *Colour Index* (1971). Seven of these, for which 1971 U.S. production data are available, are listed in Table 2.7. In addition, about 87,000 kg of a 3,3'-dimethoxybenzidine-based pigment, Pigment Blue 25 (CI 21180), was also manufactured in the United States in 1971 (U.S. Tariff Commission, 1972, cited in International Agency for Research on Cancer, 1974*a*). 3,3'-Dimethoxybenzidine-based dyes and pigments are used primarily to color leather, paper, plastics, rubber, and textiles.

TABLE 2.7. SOME DYES MANUFACTURED FROM 3,3'-DIMETHOXYBENZIDINE IN 1971

Colour Index No.	Dye	Production (10^3 kg)
24401	Direct Blue 218	479
21160	Pigment Orange 16	153
24410	Direct Blue 1	136
24400	Direct Blue 15	94
24140	Direct Blue 8	64
24411	Direct Blue 76	53
23155	Direct Blue 98	39

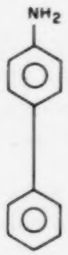
Source: Adapted from U.S. Tariff Commission, 1972, cited in International Agency for Research on Cancer, 1974*a*, p. 43.

Smaller quantities of 3,3'-dimethoxybenzidine are used in applications unrelated to dyes or pigments. The most important of these is probably the manufacture of 3,3'-dimethoxy-4,4'-diphenylene diisocyanate, an ingredient in isocyanate-based adhesive systems and a component of polyurethane elastomers. 3,3'-Dimethoxy-4,4'-diphenylene diisocyanate is prepared by dissolving the hydrochloride salt of 3,3'-dimethoxybenzidine in an aromatic solvent and heating the resulting solution with phosgene (Woolrich and Rye, 1969). The annual rate of production for this compound is not known but is estimated to be less than 500,000 kg (International Agency for Research on Cancer, 1974*a*, p. 43). 3,3'-Dimethoxybenzidine is also used in analytical chemistry to detect and determine cobalt, copper, gold, vanadium, thiocyanate, and nitrite (Welcher, 1947, p. 342).

2.2.4.3 Biochemical Reactions and Metabolites — Laham (1971) fed the monosubstituted compound 3-methoxybenzidine to rats and detected 3-methoxy-4-amino-4'-acetamidobenzidine in freshly collected urine. In vivo acetylation of this amine in the 4' rather than the 4 position indicates steric hindrance from the methoxy group in the 3 position.

2.2.5 4-Aminobiphenyl

4-Aminobiphenyl is a derivative of biphenyl rather than benzidine. It is discussed here because of its structural and chemical similarities to benzidine and its established carcinogenicity in animals and humans (*Federal Register*, 1974). The elemental and structural composition of 4-aminobiphenyl is indicated by the formulas $C_{12}H_{11}N$ and

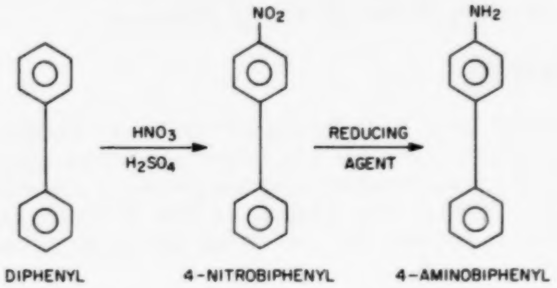


The *Chemical Abstracts* identification number of 4-aminobiphenyl is 92671. It is also known as 4-biphenylamine, *p*-aminobiphenyl, *p*-aminodiphenyl, 4-aminodiphenyl, *p*-biphenylamine, *p*-phenylaniline, and xenylamine.

2.2.5.1 Physical and Chemical Properties — 4-Aminobiphenyl is a colorless, crystalline compound that darkens on oxidation. It has a molecular weight of 169.2, a boiling point of 191°C (15 torr), and a melting point of 53°C to 54°C (Weast, 1970). 4-Aminobiphenyl is very slightly soluble in cold water and is soluble in hot water and nonpolar solvents and in lipids. Like benzidine (Section 2.2.1.2), it forms salts with mineral acids, is oxidized by air, and its amino group is diazotized, acetylated, and alkylated (International Agency for Research on Cancer, 1972).

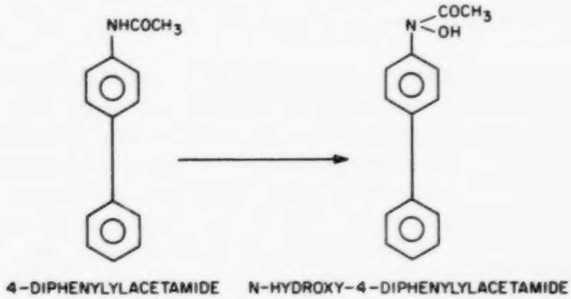
2.2.5.2 Occurrence, Synthesis, and Use — 4-Aminobiphenyl does not occur naturally; however, it can form inadvertently as an impurity during the synthesis of certain aromatic amines. In fact, 4-aminobiphenyl was first isolated in 1862 from residues remaining after the distillation of crude aniline (Hueper and Conway, 1964, p. 265). The formation of 4-aminobiphenyl in aniline is probably due to the reduction of 4-nitrobiphenyl produced by the nitration of diphenyl contained in impure benzene. Occurrence of 4-aminobiphenyl in aniline synthesized in modern plants has been greatly minimized by improved distillation techniques (International Agency for Research on Cancer, 1974*a*, p. 114).

4-Aminobiphenyl was produced on a large scale in the United States from 1935 to 1955 for use as a rubber antioxidant. It was prepared by nitrating diphenyl and treating the resulting intermediate with a mild reducing agent:

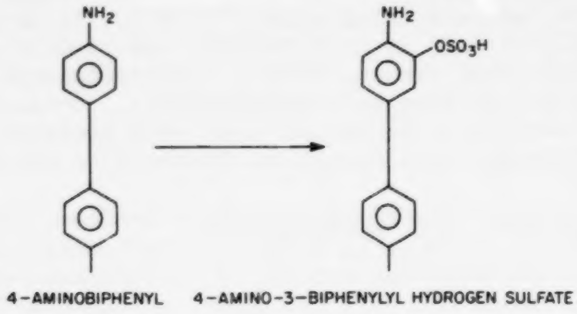


Production of the compound in the United States is thought to have ceased following the general recognition in 1955 of its carcinogenicity (International Agency for Research on Cancer, 1972, p. 74). However, it still is being manufactured, but on a smaller scale than formerly (Haley, 1977).

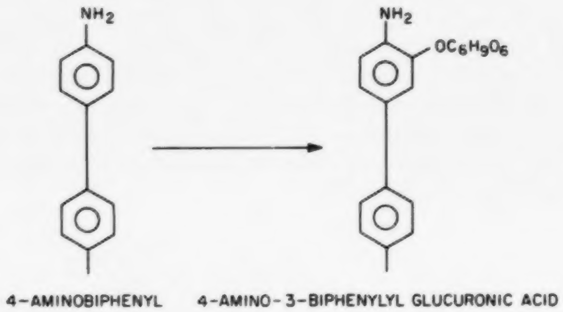
2.2.5.3 Biochemical Reactions and Metabolites — Studies seeking 4-aminobiphenyl metabolites have been performed by several investigators. Miller et al. (1961) observed that rats convert 4-diphenylacetamide to the N-hydroxy derivative:



Rabbit liver microsomes also metabolize 4-acetamidobiphenyl in this manner (Booth and Boyland, 1964). In dogs, 4-aminobiphenyl is metabolized to 4-amino-3-biphenylyl hydrogen sulfate (Bradshaw and Clayson, 1955),

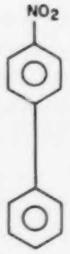


and 4-amino-3-biphenylyl glucuronic acid (Gorrod, 1971),



2.2.6 4-Nitrobiphenyl

4-Nitrobiphenyl is also a derivative of biphenyl rather than benzidine. Like 4-aminobiphenyl, it has structural and chemical similarities to benzidine and is an established carcinogen in dogs (*Federal Register*, 1974). The composition and structure of 4-nitrobiphenyl are indicated by the formulas $C_{12}H_9NO_2$ and

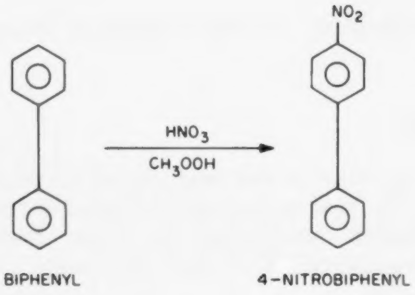


The *Chemical Abstracts* identification number of 4-nitrobiphenyl is 92-93-3. Other names for the compound are *p*-nitrobiphenyl, *p*-nitrodiphenyl, 4-nitrodiphenyl, PNB, 4-phenyl-nitrobenzene, and *p*-phenyl-nitrobenzene.

2.2.6.1 Physical and Chemical Properties — 4-Nitrobiphenyl occurs as white to yellow needles which have a molecular weight of 199.2, a boiling point of 340°C, and a melting point of 114°C. It is almost insoluble in water, slightly soluble in cold alcohol, and readily soluble in benzene, chloroform, and ether (Weast, 1970). 4-Nitrobiphenyl is easily reduced both in vitro and in vivo to 4-aminobiphenyl. *Ortho*-hydroxylation occurs on prolonged warming of 4-nitrobiphenyl with powdered potassium hydroxide in benzene (International Agency for Research on Cancer, 1974*a*).

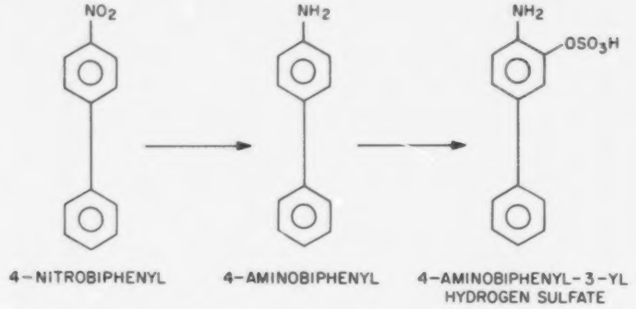
2.2.6.2 Occurrence, Synthesis, and Use — 4-Nitrobiphenyl does not occur naturally. Prior to 1900, it formed inadvertently during the commercial preparation of nitrobenzene because of the presence of biphenyl in impure benzene. This source of 4-nitrobiphenyl has apparently been eliminated by improved distillation techniques which completely remove biphenyl from the benzene feedstock (International Agency for Research on Cancer, 1974*a*).

Presently, there appears to be no large-scale industrial production of 4-nitrobiphenyl in the United States. The only known commercial application of the compound is as a chemical intermediate in the preparation of 4-aminobiphenyl (Section 2.2.5). When use of the latter material as a rubber antioxidant ceased in 1955, the commercial production of 4-nitrobiphenyl also stopped (Deichmann et al., 1958); however, small amounts are still manufactured for research and pilot plant use. The method used to prepare the 4-nitrobiphenyl consumed between 1934 and 1955 has not been reported (International Agency for Research on Cancer, 1974*a*). An obvious technique involves the nitration of biphenyl:



Synthesis and use of 4-nitrobiphenyl is prohibited in the United Kingdom under the Carcinogenic Substances Regulations, 1967, Statutory Instrument No. 487. The preparation and use of 4-nitrobiphenyl in the United States is regulated by standards set by the Occupational Safety and Health Administration (*Federal Register*, 1974).

2.2.6.3 Biochemical Reactions and Metabolites — Rats metabolically reduce 4-nitrobiphenyl to 4-aminobiphenyl and 4-aminobiphenyl-3-yl hydrogen sulfate (Laham, 1960):



A similar reduction of 4-nitrobiphenyl to 4-aminobiphenyl occurs in vitro if rat liver enzymes, the necessary cofactors, and anaerobic reaction conditions are present (Uehleke and Nestel, 1967).

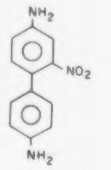
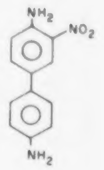
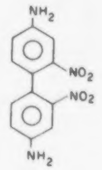
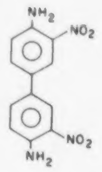
2.2.7 Other Benzidine Congeners

In addition to the benzidine compounds discussed in the preceding sections, many other substituted products are possible. Nitro, alkoxy, sulfonic acid, and carboxylic acid derivatives are the most important. Few, if any, carcinogenic or metabolic studies have been performed with these materials. Their general characteristics are summarized in Table 2.8.

2.3 ANALYSIS FOR BENZIDINE

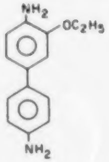
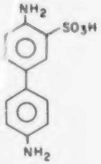
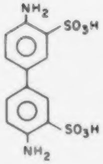
Although alarm over "aniline tumors" was expressed by Rehn as early as 1895, general concern over the carcinogenic role of benzidine and its congeners did not develop for nearly half a century (Spitz, Maguigan, and Dobriner, 1950). Approved analytical methods for determining benzidine were first established by the Association of British Chemical Manufacturers in 1954 (Butt and Strafford, 1956). These colorimetric methods were used, with innovations, for almost all of the quantitative analyses of benzidine which were subsequently reported. Recently, the manufacture and use of benzidine and 3,3'-dichlorobenzidine in this country became subject to the regulations of the Williams-Steiger Occupational Safety and Health Act of 1970 as amended in the *Federal Register*, January 29, 1974. Analytical techniques or instruments for environmental monitoring are not specified in the adopted standards, but the administration requested the National Institute for Occupational Safety and Health to develop qualitative and quantitative methods for determining the listed carcinogens (*Federal Register*, 1974). Approved methods for determining benzidine have not yet been published, but an interim procedure (Section 2.3.2.3) was suggested (*Federal Register*, 1975). In the absence of formally sanctioned analytical procedures, the discussions which follow reflect the existing state of the art as described in the current literature.

TABLE 2.8. OTHER BENZIDINE CONGENERS

Compound	Empirical formula	Structural formula	Molecular weight	Form and color	Melting point (°C)	Use	Method of preparation
2-Nitrobenzidine	$C_{12}H_{11}N_3O_2$		229.2	Red needles	143	Dye intermediate	Nitration of benzidine sulfate in sulfuric acid
3-Nitrobenzidine	$C_{12}H_{11}N_3O_2$		229.2	Red crystals	208-210		Careful reduction of 4-amino-3,4'-dinitrobiphenyl
2,2'-Dinitrobenzidine	$C_{12}H_{10}N_4O_4$		274.2	Yellow crystals	214	Dye intermediate	Nitration of benzidine in excess sulfuric acid
3,3'-Dinitrobenzidine	$C_{12}H_{10}N_4O_4$		274.2	Red needles	281-282	Dye intermediate	Hydrolysis of its N,N'-diacetyl or N,N'-di-*p*-toluene sulfonyl derivative

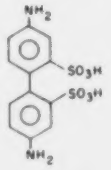
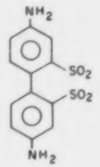
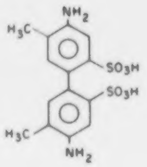
(continued)

TABLE 2.8 (continued)

Compound	Empirical formula	Structural formula	Molecular weight	Form and color	Melting point (°C)	Use	Method of preparation
3-Ethoxybenzidine	$C_{14}H_{16}N_2O$		228.3	Shiny, flat needles	134-135		Coupling benzenediazonium chloride with phenol-4-sulfonic acid, followed by ethylation of the hydroxyl group, reduction to the hydrazo compound, rearrangement with mineral acid, and desulfonation with dilute acid at 170°C
4,4'-Diamino-3-biphenylsulfonic acid	$C_{12}H_{12}N_2O_3S$		264.3			Dye intermediate	Heating benzidine sulfate to 170°C for 24 hr, extracting the product in alkali, and precipitating the monosulfonic acid with acetic acid
Benzidine-3,3'-disulfonic acid	$C_{12}H_{12}N_2O_6S_2$		344.4	Small, white plates		Dye intermediate	Baking benzidine sulfate with excess sulfuric acid at 210°C for 36 to 48 hr, extracting the product in alkali, and precipitating the product with mineral acid

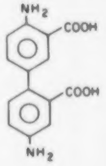
(continued)

TABLE 2.8 (continued)

Compound	Empirical formula	Structural formula	Molecular weight	Form and color	Melting point (°C)	Use	Method of preparation
Benzidine-2,2'-disulfonic acid	$C_{12}H_{12}N_2O_6S_2$		344.4	Prisms		Dye intermediate	Reducing *m*-nitrobenzenesulfonic acid with zinc dust and caustic soda at 70°C, followed by neutralization, further reduction with zinc at 80°C, filtration, and rearrangement in mineral acid at 20°C
Benzidine sulfone	$C_{12}H_{10}N_2O_2S$		246.3	Light yellow plates	>350	Dye intermediate	Treating benzidine sulfate with excess 20% oleum 100°C
o-Tolidine-6,6'-disulfonic acid	$C_{14}H_{16}N_2O_6S_2$		372.4	Needles		Dye intermediate	Reducing 2-nitrotoluene-4-sulfonic acid with zinc and caustic soda, followed by rearrangement with mineral acid

(continued)

TABLE 2.8 (continued)

Compound	Empirical formula	Structural formula	Molecular weight	Form and color	Melting point (°C)	Use	Method of preparation
Benzidine-3,3'-dicarboxylic acid	$C_{14}H_{12}N_2O_4$		272.3	Needles	275	Dye intermediate	Reducing *o*-nitrobenzoic acid with zinc dust and alkali at 100°C to 105°C, precipitating the hydrazo compound with acetic and hydrochloric acids, and rearranging the product in concentrated hydrochloric acid at 95°C to 100°C

Source: Compiled from Lurie, 1964, pp. 416-419.

2.3.1 Sampling and Sample Preparation

The melting points of benzidine and its common derivatives are well above ambient environmental temperatures; consequently, these compounds occur in the undissolved state as aerosols, dusts, or crystals and in dissolved form as aerosols, aqueous solutions, or organic solutions. The principal considerations involved in handling the various sample types are discussed in the following sections.

2.3.1.1 Benzidine in Air — Formerly, benzidine and its derivatives were manufactured and used in open systems which permitted loss of the compound to the atmosphere, the worker, and the work site. In some operations, average concentrations in the air of 2.5 to 17.6 mg/m^3 were observed (Akiyama, 1970; Zavon, Hoegg, and Bingham, 1973). Under regulations adopted in the United States in 1974, only closed systems are permitted. Loss of benzidine or other regulated carcinogens to the atmosphere is expected to be reduced to such an extent that analytical techniques having a sensitivity at the parts-per-billion level will be needed for monitoring (Anonymous, 1974). However, some exposure by inhalation can occur when equipment is being cleaned (Haley, 1975).

Another source of exposure can occur in the use by the general public of pressurized spray containers of paints, lacquers, and enamels containing benzidine yellow, an azo dye derived from 3,3'-dichlorobenzidine. The various uses of this derivative of benzidine are noted in Section 2.2.2.2. The amount of air contamination and dispersion of benzidine yellow afforded by the use of aerosol products is not known.

Benzidine in the air has previously been collected with standard Greenburg-Smith impingers (Figure 2.4) containing hydrochloric acid (International Union of Pure and Applied Chemistry, 1962; Zavon, Hoegg, and Bingham, 1973) or water (Glassman and Meigs, 1951) and with high-volume vacuum samplers equipped with fiberglass filters (Akiyama, 1970; Zavon, Hoegg, and Bingham, 1973). When necessary, the collected sample was dissolved in hydrochloric acid or extracted with diethyl ether in preparation for analysis by spectrophotometry. Under the new federal regulations, highly efficient samplers will be required in many instances since the level of contamination will be greatly reduced relative to previously existing conditions. In this case, countercurrent scrubbers, spray columns, and multistage impingers can be used. Some examples of dry, cascade impingers are shown in Figure 2.5. Sampling devices and procedures for collecting dusts from the air are further discussed in the *Air Pollution Manual* (American Industrial Hygiene Association, 1972) and by Hendrickson (1967).

2.3.1.2 Benzidine in Wastewaters — Wastewaters can be contaminated with benzidine or its congeners not only by direct addition of these substances through careless or accidental operating procedures during manufacture, but also by the discharge of benzidine-coupled dyes. These dyes are subsequently converted to the parent amine by reductants such as sulfur dioxide or hydrogen sulfide which are present in, or are later added to, the wastewater (Takemura, Akiyama, and Nakajima, 1965). Samples are normally taken in glass or polyethylene bottles. No data are available concerning storage

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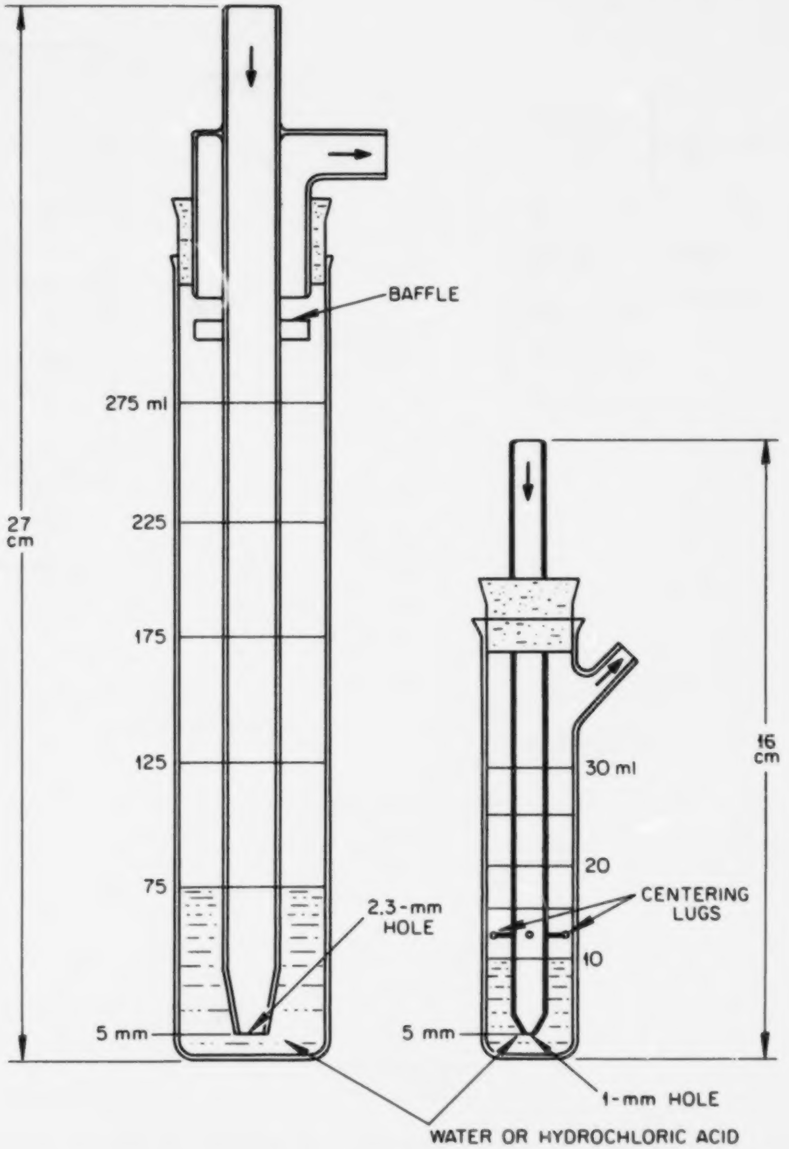

Figure 2.4. Early version of all glass Greenburg-Smith and midget impingers. Source: Adapted from American Industrial Hygiene Association, 1972, Figure 9-22, p. 133. Reprinted by permission of the publisher.

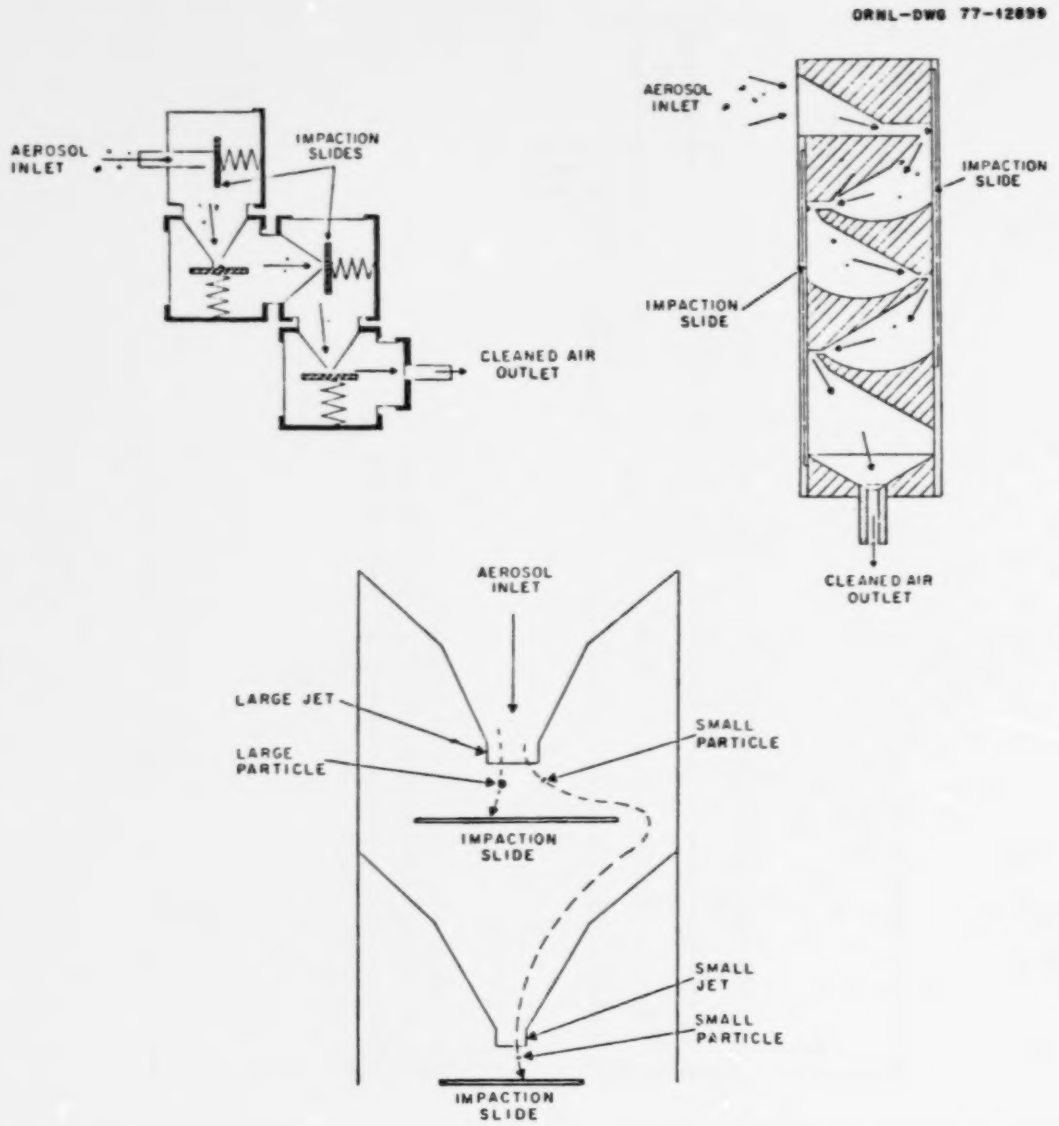

Figure 2.5. Types of dry, cascade impingers. Source: American Industrial Hygiene Association, 1972, Figure 9-23, p. 134. Reprinted by permission of the publisher.

losses. Samples of raw sewage, secondary effluents, rivers, and industrial waste streams usually require pretreatment prior to analysis for benzidine (Figure 2.6). Typically, the sample is adjusted to pH 11 by addition of sodium hydroxide, filtered to remove suspended solids, and extracted with diethyl ether. For colorimetric analyses, the resulting solution is extracted with hydrochloric acid, followed by neutralization with sodium hydroxide. If the sample is to be analyzed chromatographically, a second ether extraction and a concentration step are performed. Jenkins and Baird (1975) reported photodecomposition of benzidine during concentration of the final ether phase but observed that this can be prevented by the addition of methanol.

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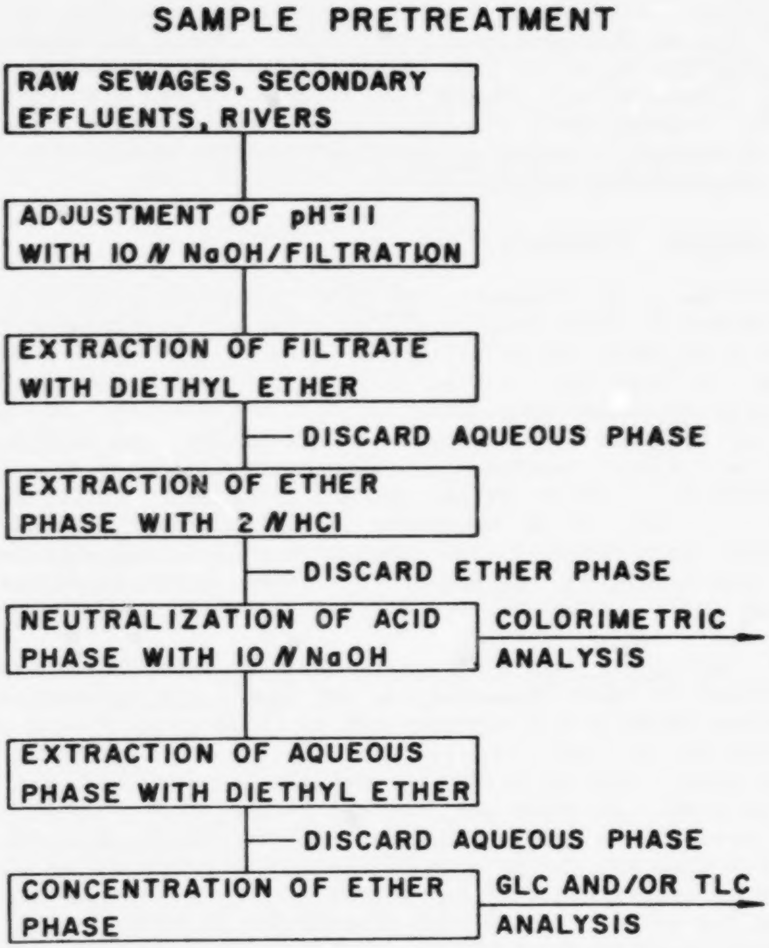
SAMPLE PRETREATMENT
RAW SEWAGES, SECONDARY EFFLUENTS, RIVERS
ADJUSTMENT OF pH≅11 WITH 10 N NaOH/FILTRATION
EXTRACTION OF FILTRATE WITH DIETHYL ETHER
DISCARD AQUEOUS PHASE
EXTRACTION OF ETHER PHASE WITH 2 N HCl
DISCARD ETHER PHASE
NEUTRALIZATION OF ACID PHASE WITH 10 N NaOH
COLORIMETRIC ANALYSIS
EXTRACTION OF AQUEOUS PHASE WITH DIETHYL ETHER
DISCARD AQUEOUS PHASE
CONCENTRATION OF ETHER PHASE
GLC AND/OR TLC ANALYSIS

Figure 2.6. Procedure for pretreatment of sample prior to analysis by gas-liquid chromatography (GLC), thin-layer chromatography (TLC), or colorimetry. Source: Jenkins and Baird, 1975, Figure 2, p. 441. Reprinted by permission of the publisher.

2.3.1.3 Benzidine in Biological Media — Since benzidine is primarily a bladder carcinogen, urine is by far the most common biological medium in which benzidine is sought; however, tissue and fecal samples are also occasionally analyzed. Urine samples should be freshly voided. If immediate analysis is not feasible, the samples should be refrigerated or frozen to avoid loss of benzidine, which occurs after only a few hours at room temperature (Sciarini and Mahew, 1955). Typically, urine samples are alkalized with sodium hydroxide to pH 8.5 to 11, filtered or centrifuged, and extracted one or more times with diethyl ether or an ethyl acetate-acetone mixture. When concentration of the sample is necessary, the organic phase is evaporated. The resulting organic extract can be used directly in determining benzidine by chromatographic techniques or it can be treated with an aqueous mineral acid solution for colorimetric analysis (Glassman and Meigs, 1951; Sciarini and Mahew, 1955). Tissues are usually homogenized and extracted with acetone. After evaporation of the solvent, the residues are washed with hot glacial acetic acid and the resulting solution is cooled and filtered. This liquid is then analyzed for benzidine by the preferred colorimetric or chromatographic technique. Typically, fecal samples are homogenized and extracted with water. After centrifugation, the aqueous phase is extracted with ether; the latter is washed with aqueous sodium hydroxide, concentrated, and back-extracted with hydrochloric acid. The resulting aqueous solution is then analyzed for benzidine by the preferred method (Sciarini and Meigs, 1961*a*).

2.3.2 Methods of Analysis

Benzidine, its congeners, and their metabolites can be determined in environmental and biological media by a variety of procedures. Those which are currently important or which show promise of future usefulness are described in this section. The performance and limitations of each method are emphasized rather than minute operational details. The methods are summarized in Table 2.9. Sensitivity, precision, and accuracy vary not only among different methods, but also among various models of analytical instruments and among different operators (Karasek, 1975); the tabulated data should therefore be considered representative rather than definitive. Performance data obtained from laboratories concerned with developing a new method usually reflect optimized conditions. Interlaboratory comparisons, when they exist, offer more realistic evaluations of the various methods.

2.3.2.1 Gas-Liquid Chromatography — Gas-liquid chromatography is an analytical process in which components of the sample are physically partitioned between two phases — a stationary bed of large surface area and a gas which percolates through and along the stationary bed. Typically, the stationary bed is a finely divided column packing which is covered with a suitable liquid sorbent. An inert gas, such as helium, argon, or nitrogen, is usually used as the carrier for the volatile phase. When the sample is introduced into the chromatographic column, the unadsorbed carrier gas moves the various constituents of the sample through the column at a rate determined by the interaction of each constituent with the liquid sorbent. Since each constituent of the sample has a slightly different affinity for the sorbent, each fraction of the sample usually emerges from a well-designed column completely resolved from other components after the passage

TABLE 2.9. METHODS FOR DETERMINING BENZIDINE

Analytical method	Important applications	Limit of detection	Precision (relative standard deviation)	Accuracy (relative error)	Interfering substances	Selectivity	Comments
Gas-liquid chromatography	Air, water, urine, blood	2-3 μg/liter (wastewater)^a				Substituted benzidine compounds which interfere in other methods can usually be separated and quantized if programmed-temperature operation is used.	Method of choice for determining benzidine and its congeners
Thin-layer chromatography	Simple, inexpensive method of separating complex mixtures of benzidine, its metabolites, and congeners; basically a qualitative procedure	0.4-0.5 μg (5-μl spot)^a				Selective for benzidine	Often used to identify the metabolites of benzidine and its congeners but only infrequently to quantify such results
Spectrophotometry (chloramine-T method)	Air, water, urine, blood	0.2 μg/liter (1-liter sample of wastewater)^b	5.1% (1.8 μg/liter of river water)^b	31% (1.8 μg/liter of river water)^b	Organic bases	Compounds similar to benzidine (e.g., *dichlorobenzidine*, *o*-tolidine, and dianisidine) interfere with quantification.	Sample color fades; must be measured rapidly and reproducibly for consistent results; nevertheless, popular procedure
Spectrophotometry (diazo dye method)	Air, water, biological samples	5 ppb (wastewater)^a 100 ppb (natural waters)^c	0.5%-6% (20-100 μg amine)	5%-30% (unchlorinated wastewater)	Hypochlorous acid (from chlorinated wastewaters)	Selective for aromatic amines in general but does not distinguish benzidine from its derivatives or many other aromatic amines	Frequently used

(continued)

TABLE 2.9 (continued)

Analytical method	Important applications	Limit of detection	Precision (relative standard deviation)	Accuracy (relative error)	Interfering substances	Selectivity	Comments
Spectrophotofluorometry	Trace analysis of benzidine, 3,3'-dimethoxybenzidine and their dihydrochloride salts in microbiological growth media, potable water and wastewaters, human urine, and rat blood	2 ng/ml (as amine)[d] 10 ng/ml (as salt)[d]	0.0%-5.0% (0.10 ppm), biomedia[d] 0.0%-1.2% (1.0 ppm), biomedia[d] 0.0%-5.0% (20 ppb), wastewater[d] 1%-25% (10 ppm), rat blood[d] 5%-18% (20 ppb) human urine[d]	32% (0.10 ppm), biomedia[d] 20% (1.0 ppm), biomedia[d] 25% (20 ppb), (wastewater)[d] 86% (10 ppm), rat blood without hydrolysis[d] 81% (10 ppm), rat blood with alkaline hydrolysis[d] 37% (10 ppm), rat blood with acid hydrolysis[d] 10% (20 ppb), human urine without hydrolysis[d] 32% (20 ppb), human urine with alkaline hydrolysis[d]	None reported	Good for benzidine, 3,3'-dimethylbenzidine, 3,3'-dimethoxybenzidine, and their salts when present singly	Although not adequately tested under field conditions, appears attractive for analysis of trace levels of these compounds in wastewater, potable waters, urine, blood, and selected biological media

[a] Jenkins and Baird, 1975.
[b] Longbottom, 1974.
[c] El-Dib, 1971.
[d] Bowman, King, and Holder, 1976.

of a characteristic volume of carrier gas. Under standardized operating conditions, each component can be identified by its characteristic elution time. The composition of the original sample is determined by identifying and measuring each component. Various kinds of detectors are available for quantifying the fractions; electron capture, flame ionization, and thermal conductivity types are commonly used. A schematic diagram of a typical system is shown in Figure 2.7.

Unlike colorimetric methods, gas-liquid chromatography readily distinguishes individual components of aromatic amine mixtures, including closely related derivatives. For example, Jenkins and Baird (1975) used gas-liquid chromatography to analyze samples of wastewater containing benzidine, 3,3'-dimethoxybenzidine, diphenylamine, and 1-napthylamine in the parts-per-billion concentration range. Their analytical system consisted of a Perkin-Elmer Model 900 gas chromatograph equipped with dual 6 ft x 1/8 in. inside diameter glass columns packed with a mixture of OV-17 (4.7%), QF-1 (5%), and DC-200(0.5%) on 80/100 mesh Gas-chrom Q. Dual flame ionization detectors were operated in the reference/sample mode and nitrogen was used as the carrier gas. Ten microliters of prepared sample (Section 2.3.1.2) were analyzed either isothermally at 200°C or programmed from 150°C to 250°C. Detection limits for benzidine were 0.1 mg/liter with the former method and 0.5 mg/liter with the latter mode of operation. Although programmed thermal operation gave decreased sensitivity for benzidine when compared with isothermal analysis, resolution was improved and complete separation of benzidine, 3,3'-dimethoxybenzidine, diphenylamine, and 1-napthylamine was obtained (Figure 2.8).

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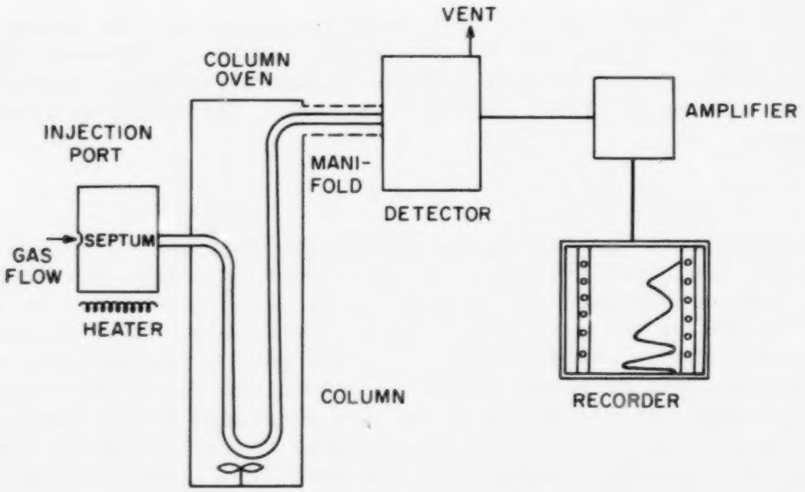


Figure 2.7. Schematic diagram of a gas chromatograph. Source: Adapted from Environmental Instrumentation Group, 1973.

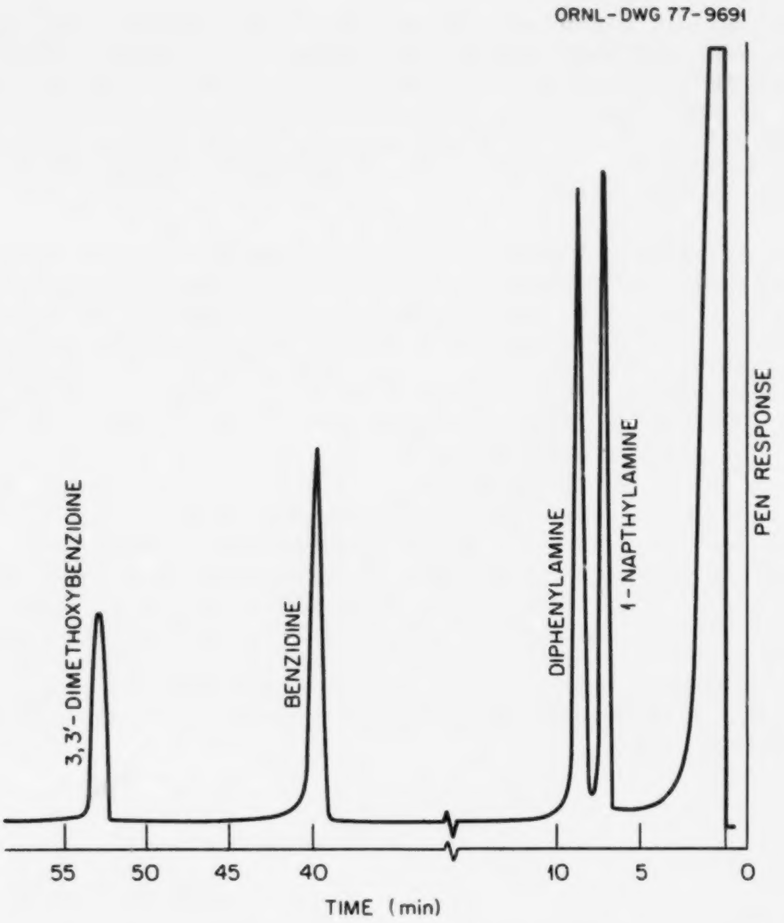


Figure 2.8. Resolution of four aromatic amines by the column mixture OV-17 (4.7%), QF-1 (5.0%), and DC-200 (0.5%) on 80- to 100-mesh Gas-chrom Q, using PTGC (150-250°C at 3°/min, holding 12 min at 150°C). Source: Adapted from Jenkins and Baird, 1975, Figure 1, p. 440. Reprinted by permission of the publisher.

Published data concerning the chromatographic determination of benzidine and its congeners are thus far very limited. Standardized procedures have not been established and statistics by which the precision and accuracy of the method can be evaluated are unavailable. Nevertheless, the superior sensitivity and selectivity of the gas chromatographic method make it very attractive; it seems likely to become the method of choice for determining mixtures of benzidine and its congeners in the parts-per-billion concentration range. The method is suitable for all types of samples if prescribed preliminary preparations are performed. The principles and analytical applications of gas chromatography are discussed extensively by Pauschmann and Bayer (1974, pp. 143-165) and by Krugers (1968).

2.3.2.2 Thin-Layer Chromatography — Thin-layer chromatography (TLC) is a separation technique in which the solution to be analyzed is deposited on a sorbent surface along which it is caused to migrate by a mobile liquid phase. As the sample moves, the various components of the sample interact with the sorbent surface in a characteristic manner and usually fractionate into distinct spots or zones which can be identified by comparison with standard chromatograms. For analysis of benzidine, a typical sorbent surface is Silica Gel G (0.25-mm thickness) or a sheet of prepared paper such as Whatman No. 1. After the sample is deposited, the chromatogram is developed by enclosing the sorbent layer in a cylindrical or sandwich-type chromatographic tank and allowing a prepared solvent to diffuse through the sorbent surface by capillary action. Usually, the direction of diffusion is upward. The solvent is a liquid or, more frequently, a mixture of liquids chosen on the basis of a trial performance. For a given sample, the more polar the solvent, the greater will be the distance of migration. After components of the sample have migrated sufficiently, usually about 10 cm above the spot origin, development is stopped and the spots are made visible by irridation with ultraviolet light or by spraying with a suitable reagent. For a given system, identification of particular compounds is given by characteristic retardation factor (R_f) values, the ratio of the distances traveled by the component and the solvent. Frequently, more positive identification is obtained by spraying the separated components with reagents which give characteristic identifying colors.

In a recent application of TLC to the determination of benzidine in wastewaters, Jenkins and Baird (1975) used 0.25-mm-thick Silica Gel G for the adsorbent, benzene-methanol (95/5) for the solvent, and ultraviolet light or 0.01% ethanolic fluorescein dye for visualization. They observed the following R_f values: diphenylamine, 85; 1-napthylamine, 70; 3,3'-dimethylbenzidine, 51; and benzidine, 37. Detection limits were 0.4 to 0.5 μg when an initial 5-μl spot was used. A similar TLC system also was described by Jakovljevic, Zynger, and Bishara (1975) for the determination of trace amounts of 4-aminodiphenyl present in 2-aminodiphenyl. The free amines were isolated by TLC, extracted from the plates, and determined fluorometrically. More recently, Holder, King, and Bowman (1976) utilized Silica Gel GF (0.25-mm-thick) to obtain TLC R_f values in ten different solvent systems. Diphenyl was used as the reference compound. The TLC system used by these authors is thus sufficiently selective to isolate benzidine from the other components of the sample, but the overall sensitivity of the method is poor.

Laham, Farant, and Potvin (1970) studied a variety of TLC systems to isolate benzidine, 4,4'-dinitrobiphenyl, and the most important biotransformation products of these compounds from human and animal urine. They used Whatman No. 1 paper sheets, ten different solvents (Table 2.10), and five different detection reagents (Table 2.11) in their work. The characteristic identifying colors of benzidine, 4,4'-dinitrobiphenyl, and their metabolites are shown in Table 2.12; the R_f values for each compound and each solvent are given in Table 2.13. The results show that the chromatographic behavior of the various compounds is related to their structure and that the migration trends of the various compounds in the selected solvents can be used to identify the biotransformation products of benzidine and 4,4'-dinitrobiphenyl.

TABLE 2.10. SOLVENTS USED FOR CHROMATOGRAPHIC SEPARATION OF 4,4'-DINITROBIPHENYL, BENZIDINE, AND THEIR RESPECTIVE METABOLIC PRODUCTS

Solvent system	Volume to volume ratio
n-Butanol, water	1:1
n-Butanol, acetic acid, water	4:1:5
n-Butanol, ethanol, *N* NH_4OH	2:1:1
sec-Butanol, 3% NH_4OH (in water)	3:1
Toluene, methanol, water	4:3:1
Cyclohexane, *tert*-butanol, pyridine, water	16:2:2:1
Ligroine, toluene, methanol, *N* NH_4OH	33:17:30:20
Ligroine, benzene, methanol, *N* NH_4OH	33:17:40:20
Ligroine, methanol, water	9:2:1
Ligroine, methanol, water	20:17:3

Source: Adapted from Laham, Farant, and Potvin, 1970, Table 1, p. 17. Reprinted by permission of the publisher.

TABLE 2.11. COMPOSITION OF SPRAYING REAGENTS USED FOR CHROMATOGRAPHIC SEPARATION OF 4,4'-DINITROBIPHENYL, BENZIDINE, AND THEIR RESPECTIVE METABOLIC PRODUCTS

No.	Name	Reagent composition	Group(s) detected
1	Ehrlich	*p*-Dimethylaminobenzaldehyde, 2 w/v % in 95% EtOH — 8 vol HCl, 6 *N* — 2 vol	NH_2, $NHCOCH_3$
2	Gibbs	First spray: sodium carbonate, 0.125 *M* Second spray: N-2,6-trichloro-*p*-quinone imine in 100 w/v % EtOH — 0.4%	OH
3	β-Resorcylaldehyde	β-Resorcylaldehyde — 1 g Glacial acetic acid — 1 ml Ethanol (100%) — 100 ml	$NHSO_3H$, OSO_3H
4	Folin ciocalteu	First spray: Folin ciocalteu Second spray: sodium carbonate, 20 w/v %	OH
5	$FeCl_3$	Potassium ferricyanide, w/v % Ferric chloride, 1 w/v %	OH

Source: Adapted from Laham, Farant, and Potvin, 1970, Table 2, p. 18. Reprinted by permission of the publisher.

TABLE 2.12. CHROMATOGRAPHIC BEHAVIOR OF 4,4'-DINITROBIPHENYL, BENZIDINE, AND THEIR METABOLITES

Chemical	Identifying colors[a]											
	Untreated paper		With spray reagents									
			Ehrlich		Gibbs		β-Resorcylal-dehyde		Folin ciocalteu		$FeCl_3$	
	DL[b]	UV[c]	DL	UV	DL	UV	DL	UV	DL	UV	DL	UV
4,4'-Dinitrobiphenyl	NC	DBl	NC	NC	NC	DBl	NC	NC	NC	NC	NC	DBl
4-Amino-4'-nitrobiphenyl	Y	FY	Or	G-Br	Y	Br-Pi	Y	DG	Y	Br	G	Br
4-Acetamido-4'-nitrobiphenyl	FtY	FtPi	FtOr	Br-Pi	NC	NC	NC	Pi	NC	FY	NC	FY
4-Amino-4'-nitro-3-hydroxybiphenyl	Y	Br	Or	Br	G	DBl	Y	Br	Ol	FGr	Gr	Br
4-Amino-4'-nitro-3-biphenylyl potassium sulfate	Y	Br	Or	FtBr	NC	Br	FtY	G	Y	FtBr	G	Br
4-Acetamido-4'-nitro-3-biphenylyl potassium sulfate	FtY	Pi	FtOr	Br	NC	Br	NC	Pi	NC	FY	NC	Br
4,4'-Diaminobiphenyl[d]	NC	FBl	R-Or	FOr	NC	DBl	Y	FGr	Gr	DBl	G	FBl
4-Acetamido-4'-aminobiphenyl[d]	NC	NC	Or	FY	NC	FBl	FtY	FGr	Gr	FGr	Bl	FBl
4,4'-Diacetamidobiphenyl	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
4,4'-Diamino-3-biphenylyl sodium sulfate[d]	NC	FBl	Pi	FtBr	NC	DBl	Y	FGr	Gr	DBl	Bl	FBl

[a] Bl — blue; Br — brown; G — green; Gr — grey; Or — orange; Pi — pink; R — red; Y — yellow; Ol — olive; Tu — turquoise; F — fluorescent; NC — no colour; D — dark; Ft — faint.

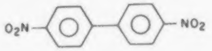
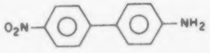
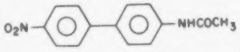
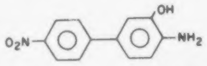
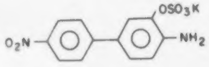
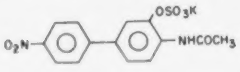
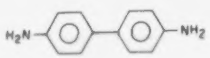
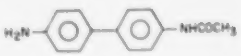
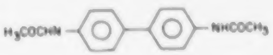
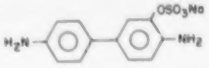
[b] Examined in daylight.

[c] Examined under ultraviolet light (2540 nm).

[d] Darkened after exposure to ultraviolet light.

Source: Adapted from Laham, Farant, and Potvin, 1970, Table 4, p. 20. Reprinted by permission of the publisher.

TABLE 2.13. RETARDATION FACTOR VALUES OF 4,4'-DINITROBIPHENYL, BENZIDINE, AND THEIR MOST IMPORTANT METABOLITES

Compound	Structure	R_f x 100 in solvent									
		1	2	3	4	5	6	7	8	9	10
1. 4,4'-Dinitrobiphenyl		91	90	88	85	88	80	88	89	57	48
2. 4-Amino-4'-nitrobiphenyl		91	89	88	86	88	58	59	57	9	9
3. 4-Acetamido-4'-nitrobiphenyl		90	90	86	86	63	39	24	15	3	0
4. 4-Amino-4'-nitro-3-hydroxybiphenyl		86	86	78	79	37	34	7	4	0	0
5. 4-Amino-4'-nitro-3-biphenylyl potassium sulfate		60	62	77	70	0	0	0	0	0	0
6. 4-Acetamido-4'-nitro-3-biphenylyl potassium sulfate		72	77	77	80	0	0	0	0	0	0
7. 4,4'-Diaminobiphenyl		84	70	79	78	43	10	2	35	4	0
8. 4-Acetamido-4'-aminobiphenyl		89	83	83	82	16	6	2	2	1	0
9. 4-4'-Diacetamidobiphenyl		83	85	83	84	22	5	0	4	0	0
10. 4,4'-Diamino-3-biphenylyl sodium sulfate		38	40	59	40	0	0	0	0	0	0

Source: Adapted from Laham, Farant, and Potvin, 1970, Table 3, p. 19. Reprinted by permission of the publisher.

From these examples, it is apparent that TLC is a convenient separation procedure which is readily adapted to the qualitative analysis of benzidine and its metabolites in environmental and biological samples. Semiquantitative estimates of sample components can also be made from thin-layer chromatograms by comparing sample spots with those on previously prepared standard chromatograms. Although convenient, this technique is not as sensitive or dependable as other methods. Better data can usually be obtained by removing identified spots from thin-layer chromatograms for quantitative determination by an appropriate conventional analytical technique. An extensive discussion of recent chromatographic developments is available in *Chromatography* (1974).

2.3.2.3 Spectrophotometry: Chloramine-T Method — The chloramine-T variant of the spectrophotometric method for determining benzidine depends on the formation of a yellow oxidation product when an acid solution of benzidine is treated with toluene-*p*-sulphonchloroamide (chloramine-T). The quantity of benzidine in the sample is determined by extracting the yellow oxidation product with an organic solvent and measuring the amount of light it absorbs under standardized conditions (Butt and Strafford, 1956; Glassman and Meigs, 1951). The color reaction is not specific for benzidine; all *p*,*p*'-diaminodiphenyl derivatives form the yellow oxidation product to some degree. Furthermore, the yellow oxidation product is not permanent; it is photosensitive and gradually fades to a colorless state, possibly a quinone complex, over a 2-hr interval. The light absorption of the sample must therefore be measured immediately after the color is developed.

In spite of these inadequacies, the chloramine-T method is convenient, and it is frequently used to determine benzidine and its derivatives. In the most recent and sensitive version of the method (Longbottom, 1974), aqueous samples are adjusted to pH 8.5 to 9.0 with sodium hydroxide or hydrochloric acid and extracted with ethyl acetate. The latter is back-extracted with hydrochloric acid and the resulting aqueous solution is refrigerated in the dark until the remaining steps of the procedure can be performed rapidly on one sample at a time. Chloramine-T is then added to the aqueous solution, with shaking, to develop the yellow oxidation product. The product is extracted with ethyl acetate, filtered, and scanned with a double-beam spectrophotometer over the spectral range of 510 to 370 nm. Benzidine is identified by its absorbance maximum at 436 nm (Figure 2.9). The spectrum of dichlorobenzidine is similar, but it has an absorbance maximum at 445 nm. The detection limit of the method is 0.2 μg/liter if 1-liter samples are used. The average relative standard deviation (n = 8) is 5.1% for analyses of 1-liter samples of river water containing 1.8 μg benzidine. The relative error under these conditions is large — 31.1%. Organic bases interfere with the analysis, as do compounds having a structure very similar to benzidine, such as dichlorobenzidine, 3,3'-dimethylbenzidine, and 3,3'-dimethoxybenzidine. This method can also be applied to benzidine or its derivatives in samples of air, urine, feces, and blood if suitable sample preparation steps are taken.

2.3.2.4 Spectrophotometry: Diazo Dye Method — This analytical method is based on the conversion of benzidine to its diazo salt and subsequent coupling to an aromatic phenol to produce a diazo dye which absorbs light

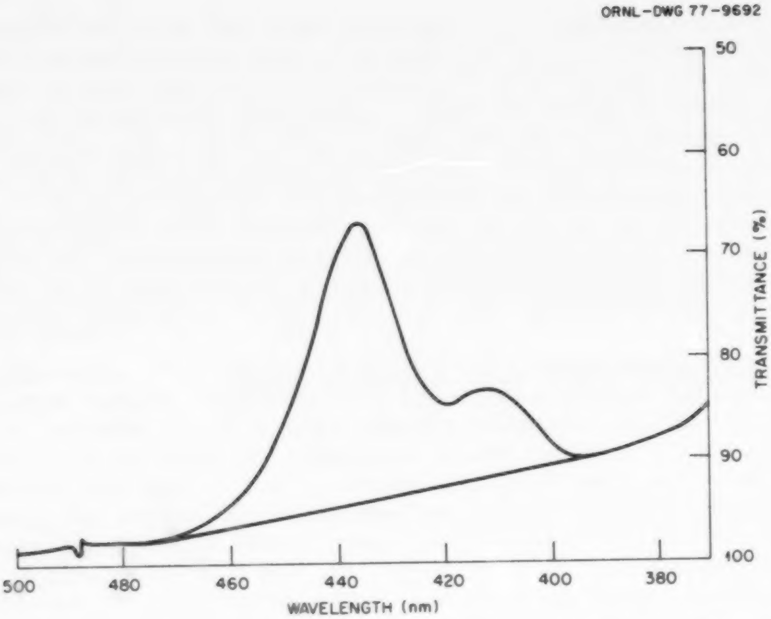


Figure 2.9. Spectrum of benzidine oxidation product. Source: Adapted from Longbottom, 1974, Figure 1.

in the visible portion of the electromagnetic spectrum. The amount of absorbed light is related to the benzidine concentration in the sample by comparison with a previously determined calibration plot.

In a typical spectrophotometric analysis of benzidine in wastewater (Jenkins and Baird, 1975), the sample is adjusted to pH 11 by addition of 10 *M* sodium hydroxide solution, filtered to remove suspended solids, diazotized with sodium nitrite and hydrochloric acid, treated with sulfamic acid to destroy excess nitrite, and coupled with resorcinol. Absorption of the resulting diazo dye is determined at 550 nm with a double-beam spectrophotometer. When 10-cm absorption cells are used, the detection limit for benzidine is about 5 ppb. For unchlorinated wastewaters, the relative error of spiked samples varies from 5% to 30%. Recovery of benzidine from chlorinated wastewater is poor, however, apparently because hypochlorous acid reacts with benzidine to yield a chloramine-type compound which does not give a positive benzidine test. Although this procedure readily distinguishes between aromatic and alkyl amines, it is not selective for benzidine in the presence of other aromatic amines; accordingly, the method is unsuitable for samples containing mixed aromatic amines. Despite these shortcomings, the diazo dye technique is a useful analytical method for determining benzidine in the absence of other aromatic amines and is frequently used for all types of samples when this condition is satisfied.

2.3.2.5 Spectrophotofluorometry — Spectrophotofluorometry is based on the measurement of fluorescence radiation emitted by a sample previously excited by ultraviolet or visible light. The emitted radiation results from the transition of the excited molecule from the first excited singlet state to the ground state; the frequency of the emitted light is therefore characteristic of the analyte. The intensity of the emission is proportional to the concentration of the analyte as well as the intensity of the exciting radiation. Because of the latter relationship, spectrophotofluorometry is inherently very sensitive; under favorable conditions, it can be four orders of magnitude more sensitive than molecular absorption spectrophotometry (Mancy, 1971, p. 70). In a typical application of spectrophotofluorometry to the determination of benzidine in environmental or biological media, samples are prepared as described in Section 2.3.1.2 or 2.3.1.3 except that methanol, rather than ether, is used as the final solvent. The prepared sample is then irradiated with monochromatic light from mercury or xenon lamps and a selected wavelength of the resulting fluoresence is measured with a photodetector tube. The intensity of the measured fluoresence is then related to the amount of benzidine in the original sample by means of a predetermined calibration curve. Bowman, King, and Holder (1976) determined benzidine, 3,3'-dimethylbenzidine, 3,3'-dimethoxybenzidine, and their dihydrochloride salts in a variety of matrices with a spectrophotofluorometer equipped with a xenon lamp, a 1P28 photodetector, and 1-cm square cells. The frequencies of the incident (λ_{Ex}) and emitted (λ_{Em}) radiations varied for each compound and are shown in Table 2.14. The relative intensities of the fluorescence of each compound are also listed. The detection limits of the amines and their salts were 2 ng/ml and 10 ng/ml respectively. Good precision was obtained in a variety of sample types; relative standard deviations of 0.0% to 5.0% were reported for trypticase soy dextrose and brain heart infusion medium containing 0.1 ppm and 1.0 ppm benzidine (Table 2.15) and wastewater containing 20 ppb benzidine (Table 2.16). Somewhat poorer results (1% to 25%) were obtained with human urine (20 ppb) and rat blood (10 ppm). The relative errors in the above analyses varied from 10% to 35%, except for the samples of rat blood, which averaged about 85%. This level of accuracy is generally adequate for all samples mentioned other than rat blood.

TABLE 2.14. WAVELENGTHS USED AND RELATIVE INTENSITIES OBSERVED DURING ANALYSIS OF BENZIDINE AND ITS CONGENERS

Compound	λ_{Ex} (nm)	λ_{Em} (nm)	Relative intensity per μg per ml
Benzidine	295	396	33.5
Benzidine•2HCl	302	410	7.45
3,3'-Dimethylbenzidine	300	384	51.8
3,3'-Dimethylbenzidine•2HCl	310	410	12.2
3,3'-Dimethoxybenzidine	312	380	64.3
3,3'-Dimethoxybenzidine•2HCl	318	422	8.25

Source: Adapted from Bowman, King, and Holder, 1976, p. 211. Reprinted by permission of the publisher.

TABLE 2.15. ANALYSIS OF TWO BIOLOGICAL GROWTH MEDIA SPIKED WITH BENZIDINE, TWO CONGENERS, AND THEIR SALTS AT 0, 0.10, and 1.0 ppm

Compound	Medium[a]	Amount added[b]		Amount recovered ($\bar{x}$ ± SE)[c]	
		(ppm)	(μg)	(ppm)	(%)
Benzidine	BHI	0	0	0.040 ± 0.000	
		0.10	1.0	0.070 ± 0.000	70.0 ± 0.0
		1.00	10.0	0.780 ± 0.004	78.0 ± 0.4
	TSD	0.00	0.0	0.019 ± 0.000	
		0.10	1.0	0.075 ± 0.000	75.0 ± 0.0
		1.00	10.0	0.787 ± 0.012	78.7 ± 1.2
Benzidine•2HCl	BHI	0.00	0.0	0.040 ± 0.000	
		0.10	1.0	0.073 ± 0.002	73.0 ± 2.0
		1.00	10.0	0.795 ± 0.000	79.5 ± 0.0
	TSD	0.00	0.0	0.019 ± 0.002	
		0.10	1.0	0.073 ± 0.001	73.0 ± 1.0
		1.00	10.0	0.778 ± 0.004	77.8 ± 0.4
3,3'-Dimethylbenzidine	BHI	0.00	0.0	0.037 ± 0.001	
		0.10	1.0	0.074 ± 0.005	74.0 ± 5.0
		1.00	10.0	0.839 ± 0.010	83.9 ± 1.0
	TSD	0.00	0.0	0.029 ± 0.004	
		0.10	1.0	0.073 ± 0.002	73.0 ± 2.0
		1.00	10.0	0.902 ± 0.009	90.2 ± 0.9
3,3'-Dimethylbenzidine•2HCl	BHI	0.00	0.0	0.045 ± 0.004	
		0.10	1.0	0.070 ± 0.002	70.0 ± 2.0
		1.00	10.0	0.777 ± 0.006	77.7 ± 0.6
	TSD	0.00	0.0	0.036 ± 0.003	
		0.10	1.0	0.064 ± 0.001	64.0 ± 1.0
		1.00	10.0	0.710 ± 0.003	71.0 ± 0.3
3,3'-Dimethoxybenzidine	BHI	0.00	0.0	0.031 ± 0.003	
		0.10	1.0	0.078 ± 0.001	78.0 ± 1.0
		1.00	10.0	0.923 ± 0.007	92.3 ± 0.7
	TSD	0.00	0.0	0.019 ± 0.001	
		0.10	1.0	0.055 ± 0.000	55.0 ± 0.0
		1.00	10.0	0.829 ± 0.008	82.9 ± 0.8
3,3'-Dimethoxybenzidine•2HCl	BHI	0.00	0.0	0.030 ± 0.003	
		0.10	1.0	0.057 ± 0.005	57.0 ± 5.0
		1.00	10.0	0.715 ± 0.002	71.5 ± 0.2
	TSD	0.00	0.0	0.027 ± 0.003	
		0.10	1.0	0.052 ± 0.001	52.0 ± 1.0
		1.00	10.0	0.770 ± 0.006	77.0 ± 0.6

[a] BHI — brain heart infusion; TSD — trypticase soy dextrose.

[b] Per 10 ml of sample.

[c] Mean and standard error from triplicate assays; spiked samples are corrected for controls. Controls and 0.10-ppm samples contained 1-g equivalent of medium per milliliter for spectrophotofluorometric reading; the 1.0-ppm samples contained 0.2-g equivalents per milliliter.

Source: Adapted from Bowman, King, and Holder, 1976, Table I, p. 216. Reprinted by permission of the publisher.

TABLE 2.16. ANALYSIS OF WASTEWATER SPIKED WITH BENZIDINE, TWO CONGENERS, AND THEIR SALTS AT 0 AND 20 ppb

Compound	Amount added[a]		Amount recovered ($\bar{x}$ ± SE)[b]	
	(ppb)	(µg)	(ppb)	(%)
Benzidine	0	0.0	4 ± 1	
	20	2.0	17 ± 0	85
Benzidine•2HCl	0	0.0	4 ± 1	
	20	2.0	15 ± 1	75
3,3'-Dimethylbenzidine	0	0.0	4 ± 1	
	20	2.0	15 ± 0	75
3,3'-Dimethylbenzidine•2HCl	0	0.0	3 ± 1	
	20	2.0	13 ± 1	65
3,3'-Dimethoxybenzidine	0	0.0	3 ± 0	
	20	2.0	16 ± 0	80
3,3'-Dimethoxybenzidine•2HCl	0	0.0	3 ± 0	
	20	2.0	14 ± 1	70

[a] Per 100 ml of sample.

[b] Mean and standard error from triplicate assays; spiked samples are corrected for controls. Samples contained 10-g equivalents of water per milliliter for spectrophotofluorometric reading.

Source: Bowman, King, and Holder, 1976, Table II, p. 217. Reprinted by permission of the publisher.

In continued research in the same laboratory (Holder, King, and Bowman, 1976), 4-aminobiphenyl was determined utilizing the same substrates and techniques. Recovery rates ranged from 81% to 95% in biological growth media and wastewater, from 45% to 95% in human urine, and from 6% to 76% in mouse whole blood. Best recovery from urine occurred without prior hydrolysis, and acid hydrolysis led to an unsuitable purple discoloration. However, best recovery from blood occurred after acid hydrolysis (67% to 72%).

Although the spectrophotofluorometric method is very specific for individual benzidine compounds because of the particular wavelengths involved, an effort by Bowman, King, and Holder (1976) to determine three amines or their three salts in admixture by measuring the relative intensities at each excitation and emission maxima was unsuccessful because of large differences in the specific responses and extensive overlap of the spectra. Further experience is needed to define the useful limits of this [illegible]lication, but the method appears attractive for the determination of tr[illegible]vels of benzidine and its congeners in a variety of environmental samples.

2.3.3 Comparison of Analytical Procedures

Although benzidine has been known since 1845 and has been used extensively in analytical chemistry to determine other substances, relatively little attention has been given to analytical procedures by which the reagent itself can be determined, particularly at the low concentrations which are of current interest. Indeed, the International Union Against Cancer concluded that "present analytical methods are satisfactory for the assessment of gross aromatic amine contamination of the human environment" but that "new techniques will have to be designed for the measurement of lower degrees of contamination" (Shubik, Clayson, and Terracini, 1970, p. 16). More recently, the U.S. Environmental Protection Agency stated, "Adequately tested methods for benzidine are not available," and recommended the chloramine-T method described in Section 2.3.2.3 as an interim method (*Federal Register*, 1975). Thus, there are no officially sanctioned analytical procedures for determining low levels of benzidine in environmental and biological samples.

The most likely candidate for official sanction appears to be gas (or gas-liquid) chromatography. Although only a limited number of gas chromatographic analyses of benzidine have been performed, the technique has already demonstrated a high degree of sensitivity, selectivity, versatility, and speed, and better performance can be expected with further use. Gas chromatography is the only analytical technique available which readily determines benzidine in the presence of its congeners. The chief disadvantages of the gas chromatographic method are its relatively high capital investment and maintenance costs and the somewhat higher level of sophistication required in the operating and maintenance personnel compared with other methods of analysis.

The determination of benzidine by spectrophotofluorometry has been even more limited than the use of gas chromatography. Nevertheless, spectrophotofluorometry is potentially important as an analytical method for benzidine and its congeners. These compounds emit distinctive fluorescent spectra by which each can be identified and quantified. The method is characterized by inherent sensitivity, simplicity, and speed. However, quantitative determinations of mixed benzidine congeners are not yet feasible because of spectral overlap and large differences in relative intensities (Bowman, King, and Holder, 1976). The importance of this deficiency depends on the composition and type of sample to be analyzed — in many instances it is not significant. In any event, marked improvement in the sensitivity and selectivity of this technique may be expected with continued use.

The diazo and chloramine-T spectrophotometric methods of analyzing benzidine and its derivatives are the oldest, most widely used methods described. Each method has benefited from a series of improvements over the years; recent versions of each method have sensitivities in the parts-per-billion range, good precision, and moderate accuracy. However, neither method distinguishes between benzidine and its congeners; the diazo technique is responsive to aromatic amines in general and the chloramine-T method responds generally to *p*,*p*'-diaminodiphenyl derivatives. In addition, the chloramine-T method is photosensitive, requiring rapid reading of the samples to prevent inaccurate results (Butt and Strafford, 1956). Despite these shortcomings, both spectrophotometric procedures are useful. For example, a modern variation of the chloramine-T method was designated by the U.S. Environmental Protection Agency as the interim method of determining benzidine (*Federal Register*, 1975).

The thin-layer chromatographic technique is a simple, frequently used qualitative procedure for separating and identifying benzidine, its congeners, and their metabolites. Although the procedure can be applied to the quantitative determination of these compounds by comparing the area or fluorescence of separated spots with that of standard chromatograms, it is relatively insensitive and is not the method of choice.

SECTION 2

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SECTION 3

BIOLOGICAL ASPECTS IN MICROORGANISMS

No data were found on the metabolism of benzidine in microorganisms. However, some duodenal bacteria, soil bacteria, and *Escherichia coli* can reduce the azo linkage of benzidine-based azo dyes to liberate benzidine (Yoshida and Miyakawa, 1973).

Antiviral activity has been demonstrated among benzidine-based dyes. Of 36 such dyes tested, 5 showed antiviral activity and 26 showed hemagglutinating activity (Dettori, 1964). No relationship occurred between the intensities of the hemagglutinating and antiviral activities. Congo Red (derived from benzidine) and benzopurpurin (derived from 3,3'-dimethylbenzidine) inhibited T_1 and T_7 phage production in a strain of *Escherichia coli* (Dettori and Neri, 1964). In this study, the dyes showed no bactericidal activity and did not inhibit respiration or anaerobic glycolysis.

Congo Red has been used as a bacteriostatic agent in the isolation of the tubercle bacillus from sputum (Frobisher, 1946) and as an indicator dye in the mixed culture growth of *Rhizobium* and related species of bacteria (Vincent, 1970).

The *Federal Register* (1976) noted that benzidine inhibited biooxidation in activated sludges at 500 mg/liter, caused some inhibition at 1 to 5 mg/liter, and was thought to be oxidized at 0.1 mg/liter or less. Howard and Saxena (1976), in reporting on work done by the Synthetic Organic Chemical Manufacturers Association in 1975, noted that Warburg tests showed benzidine inhibition of oxygen uptake at concentrations of 40 to 80 mg/liter in unacclimated sludge and at concentrations of 60 to 120 mg/liter in acclimated sludge.

Benzidine and some of its congeners have been found to be mutagenic to strains of *Salmonella typhimurium* through the use of an assay method recently developed for the testing of the mutagenic and carcinogenic potential of a wide variety of chemicals (McCann and Ames, 1976). Ames et al. (1973) first showed that benzidine was mutagenic to this bacterium, although only after it had been activated by a rat liver homogenate. Since the assay involved the detection of reversals of frameshift mutations, it was suggested that benzidine could cause frameshift mutations. More recently, Garner, Walpole, and Rose (1975) have used tester strain TA 1538 of *Salmonella typhimurium* in determining the mutagenic capability of benzidine and a number of congeners in the presence and absence of a preparation of liver mixed function oxidase. The assay measured the production of histidine prototrophs (revertants). As noted in Table 3.1, 3,3'-dichlorobenzidine and 3,3'-dichlorobenzidine sulfate (technical grade) were the only compounds which exhibited weak mutagenic activity in the absence of the liver preparation. 3,3',5,5'-Tetramethylbenzidine produced no mutagenic activity even in the presence of the liver preparation. The presence of the liver preparation caused weak mutagenic activity by 3,3'-dianisidine and strong mutagenic activity by 3,3',5,5'-tetrafluorobenzidine, 3,3'-dichlorobenzidine, and 3,3'-dichlorobenzidine sulfate.

TABLE 3.1. MUTAGENIC ACTIVITY OF BENZIDINE ANALOGUES TO *SALMONELLA TYPHIMURIUM* IN THE PRESENCE OR ABSENCE OF LIVER MIXED FUNCTION OXIDASE

Compound	Amount added (μg/plate)	Liver preparation[a]	Mutagenic activity (His^+ revertants per plate)
Benzidine	50	+	430
	100	+	640
	50	−	5
	100	−	15
3,3',5,5'-Tetrafluorobenzidine	50	+	560
	100	+	1040
	50	−	20
	100	−	29
3,3',5,5'-Tetramethylbenzidine	50	+	15
	100	+	15
	50	−	5
	100	−	9
3,3'-Dichlorobenzidine	50	+	3360
	100	+	7520
	50	−	114
	100	−	131
3,3'-Dichlorobenzidine sulfate (technical grade)	50	+	5490
	100	+	8350
	50	−	127
	100	−	129
3,3'-Dianisidine (3,3'-dimethoxybenzidine)	50	+	63
	100	+	82
	50	−	6
	100	−	11
Dimethylsulfoxide (control)		+	16
		−	8

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[a] + indicates liver homogenate present; − indicates liver homogenate absent.

Source: Adapted from Garner, Walpole, and Rose, 1975, Table 1, p. 41. Reprinted by permission of the publisher.

SECTION 3

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SECTION 4

BIOLOGICAL ASPECTS IN PLANTS

The only reference found in the literature concerning benzidine metabolism in higher plants involved the in vitro oxidation of 3,3'-diaminobenzidine by a commercial preparation of horseradish peroxidase (Barbotin and Thomas, 1974). The oxidative activity occurred when the peroxidase was in solution or immobilized in an artificial membrane. Oxidation has also been shown to occur with peroxidase from a variety of green algae, but not with peroxidases from varieties of red and brown algae (Siegel and Siegel, 1970). Diaminobenzidine oxidation in blue-green algae occurs via the respiratory and photosynthetic electron transport systems (Lauritis et al., 1975).

Diaminobenzidine is used in electron microscopy as a histochemical stain. It apparently is oxidized by the cytochromes of the mitochondrial electron transport chain, polymerizes and remains tightly bound to the oxidizing heme protein, and reacts with osmium tetroxide to give an electron-dense deposit (Öpik, 1975). Diaminobenzidine can also be used to localize sites of peroxidase and catalase activity (Nougarède, 1971; Silverberg, 1975).

No information was found in the literature on any effects to higher plants produced by benzidine exposure. However, Boney (1974) has noted that benzidine (0.3 mg/liter) provided a 12% stimulation in growth over control culture conditions in the growth of the red alga *Antithamnion plumula*.

SECTION 4

REFERENCES

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SECTION 5

BIOLOGICAL ASPECTS IN WILD AND DOMESTIC ANIMALS

No data were found concerning the metabolism of benzidine or its derivatives by wild or domestic animals. Metabolism by research animals is discussed in Section 6; there are differences among species of animals. Therefore, it is reasonable to expect some variation among diverse aquatic and terrestrial fauna.

Apparently, the only information available on the toxicity of benzidine or its derivatives to wild or domestic animals is that which has been obtained through laboratory research on a limited number of species, including the commonly used research animals. Most of this information is covered in Section 6 since it provides comparative data useful in determining the toxicity potential of these compounds to humans, including their carcinogenic potential. Some of the data are also useful in projecting the toxicity potential among wild and domestic animals, should they be exposed in their natural environment. Thus, recent bioassays of benzidine in fish have shown TL_{50} values ranging from 2.5 mg/liter for red shiner to 20 mg/liter for the fathead minnow (*Federal Register*, 1976). Pliss and Khudoley (1975) found liver damage in guppies fed 30 mg benzidine per 100 g of dry diet material. At two to four weeks of exposure, liver tissue developed foci of necrosis and fatty dystrophy. Hyperplasia of the hepatocytes occured by four to eight weeks. None of the fish had developed tumors by the 56-week termination of the experiment.

Benzidine has not been found to occur in the aquatic environment of the United States (*Federal Register*, 1976), although it has been detected in the Sumida River of Japan (Takemura, Akiyama, and Nakajima, 1965). Should contamination occur, the possibility exists for heightened toxicity among some species because of the potential of bioaccumulation within food chains. The *Federal Register* (1976) reported that bioaccumulation occurred in bluegill fish in a radiometric study using ^{14}C-benzidine. Edible tissues contained a 44-fold increase in concentration over that in water.

Benzidine injected into the blastoderm of early chick embryos at 18 to 24 hr of incubation was found by Noto (1967) to cause morphological abnormalities. The closure of the cephalic neural tube was inhibited.

SECTION 5

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SECTION 6

BIOLOGICAL ASPECTS IN HUMANS

6.1 SUMMARY

Benzidine exposure is primarily an industrial problem among workers in plants which manufacture or use benzidine and its derivatives. Uptake of these compounds is by skin absorption, inhalation, and ingestion. Skin absorption and inhalation are the major uptake routes; ingestion adds an insignificant portion, except for Japanese kimono painters who use their lips to moisten brushes dipped in benzidine dyes. Uptake is positively correlated with industrial hygiene and the particular benzidine compound to which the worker has been exposed. Volatile compounds, compounds of small particle size, and bases easily penetrate the skin. High air temperature and relative humidity also aid skin penetration.

Benzidine does not appear to accumulate in human blood or other tissues for any appreciable length of time. Studies made with warm-blooded experimental animals also showed no significant accumulation by benzidine or its derivatives. However, there appears to be rapid distribution by the blood to other tissues and temporarily increased concentrations in some organs, notably the liver, lung, and kidney, although this is not a consistent finding; findings vary with the compound tested and the test animal used. Although the usual experimental animal does not appear to accumulate benzidine in its tissues, a recent study has demonstrated a 44-fold accumulation of this compound in bluegill fish.

Benzidine and its derivatives are biotransformed into several metabolites in the animal body; the metabolites produced are species dependent. The dog differs from other animal species in that it cannot acetylate benzidine. The major benzidine metabolite in both humans and dogs appears to be 3-hydroxybenzidine. In humans, benzidine and 3,3'-dimethylbenzidine (diorthotolidine) seem to be metabolized similarly since hydroxylation of diorthotolidine occurs in the *ortho* position with respect to the amino group.

Benzidine, its derivatives, and metabolites are eliminated in the urine and feces. Biliary secretion contributes to fecal excretion. Urinary excretion in humans increases with increased exposure. There is evidence that urinary excretion in exposed workers is higher in warmer periods of the year because of increased uptake. Excretion in experimental animals varies with the compound administered and the species used. The rat, for example, excretes benzidine primarily in the feces, whereas benzidine is excreted primarily in the urine by the dog and monkey. In contrast, 3,3'-dichlorobenzidine is excreted primarily in the feces by all three animals. Bile appears to contain the same metabolites as urine, but in only one-third the quantity.

Benzidine and its derivatives produce toxic effects in humans and test animals, including the induction of cancer. In humans, strong evidence implicates benzidine and the related compound 4-aminobiphenyl as a cause of bladder cancer among workers occupationally exposed to these compounds. Although exposure to 3,3'-dimethylbenzidine, 3,3'-dimethoxybenzidine, or 3,3'-dichlorobenzidine remain suspect, there is no present evidence that bladder cancer is induced in humans. However, a recent report has suggested that 3,3'-dichlorobenzidine may be responsible for other types of tumor production in humans other than bladder cancer, and all three compounds, as well as benzidine and 4-aminobiphenyl, have produced various tumors in experimental animals. Interestingly, evidence for the induction of bladder cancer in test animals is very meager for all of these compounds except 4-aminobiphenyl. It is generally accepted that the actual carcinogenic compounds are metabolites of the parent aromatic amines. There is mounting evidence that the precarcinogen may be produced through N-hydroxylation of the parent compound, but esterification may be required as a further activation step. The sulfate and glucuronidate conjugation products of the aromatic amines may also be carcinogenic. Benzidine and its derivatives cause dermatitis, cystitis, nephritis, and hematuria in humans and various effects in test animals.

Benzidine and its derivatives also influence the activity of some enzymes. Workers exposed to benzidine had higher levels of β-glucuronidase in their urine than unexposed workers. Cessation of exposure led to more nearly normal urinary levels. Benzidine also has been shown to decrease blood phenolase activity in rabbits and catalase and peroxidase activity in rats. In vitro studies have shown that benzidine, its derivatives, and related compounds exert differing degrees of ability to reduce cytochrome *c*. This reducing power appears to relate inversely with the strength of the compounds as carcinogens. Thus, a compound such as diaminobenzidine, believed to be a weak carcinogen, displays strong reducing power, whereas a potent carcinogen such as *p*-aminobiphenyl displays weak reducing power.

6.2 METABOLISM

6.2.1 Uptake

Exposure to benzidine and benzidine derivatives is of concern mainly to employees in factories which manufacture and use these compounds. Benzidine uptake by exposed persons may be through the respiratory tract, from the air, by contaminated food into the gastrointestinal tract, and by penetration of the skin through direct contact or from contaminated clothing (Meigs, Brown, and Sciarini, 1951). All steps in benzidine synthesis as carried out by large manufacturers in the United States are now conducted in closed systems. Even under such conditions, some exposure can occur when equipment is being cleaned (Haley, 1975). There is also a question as to whether closed systems are being used by firms within the dye industry who may be manufacturing their own benzidine and derivatives (Haley, 1977).

Ingestion of benzidine or its derivatives has not been reported as a significant source of exposure with the exception of painters of silk kimonos in Japan who "point" or moisten their brushes with their lips and thereby ingest some of the benzidine dyes on the brushes (Yoshida and Miyakawa, 1973).

Skin penetration is the most important avenue of entry into the body. Both benzidine and 3,3'-dimethylbenzidine readily penetrate intact skin (Meigs, Brown, and Sciarini, 1951). 3,3'-Dichlorobenzidine, which is nonvolatile and has a large particle size, presents little inhalation hazard and less skin absorption hazard than benzidine (Gerarde and Gerarde, 1974; Rye, Woolrich, and Zanes, 1970). Barsotti and Vigliani (1952) pointed out that workers occupationally exposed in the manufacture of benzidine risk development of tumor lesions because the light, fluffy, powdery composition of the benzidine base provides for ready skin contact and absorption. The order of acute toxicity of benzidine and some of its derivatives appears to follow the order of ease of skin penetration: benzidine, 3,3'-dimethoxybenzidine, and 3,3'-dichlorobenzidine (Rye, Woolrich, and Zanes, 1970).

Meigs, Sciarini, and Van Sandt (1954) suggested that environmental conditions of high air temperature and high relative humidity influence the quantity of benzidine, 3,3'-dimethoxybenzidine, 3,3'-dichlorobenzidine, and 3,3'-dimethylbenzidine which penetrates the skin. Men who perspired freely, thus having wet skin, had higher amounts of benzidine in their urine, indicating a more rapid skin penetration, than men whose skin was less wet. Benzidine skin penetration was higher on hot days than on cool days, as indicated by increased benzidine urine concentrations during high temperatures (Table 6.1).

6.2.2 Distribution and Accumulation

No information was found on direct measurements made to determine the distribution and accumulation in human tissue of benzidine or its derivatives. However, indirect evidence afforded by the measurements of benzidine in urine samples from occupationally exposed people (Meigs, Brown, and Sciarini, 1951) indicates that no appreciable accumulation occurs with this compound.

TABLE 6.1. RELATIONSHIP BETWEEN ENVIRONMENTAL CONDITIONS AND BENZIDINE CONCENTRATIONS IN URINE OF SIX PRESSROOM WORKERS

Environmental conditions	Mean temperature (°C)	Mean relative humidity	Mean urinary specific gravity	Mean quinonizable substance expressed as benzidine (mg/liter)
Cool, dry days	23	63	1.026	0.313
	21	58	1.024	0.362
Hot, humid days	34	85	1.027	0.668
	25	78	1.025	0.895

Source: Adapted from Meigs, Sciarini, and Van Sandt, Arch. Ind. Hyg. Occup. Med., Vol. 9, Table 6, p. 129. Copyright 1954, American Medical Association.

Studies made with experimental animals, discussed below, suggest that benzidine and its derivatives are distributed fairly rapidly into various tissues following uptake but do not remain for any great length of time before elimination. Prior to elimination, these compounds or their metabolites appear to concentrate in some tissues more than others.

According to Solimskaya (1968), benzidine was rapidly absorbed within 1 hr of injection into rats. Maximum concentrations of free and bound benzidine were detected at 2 and 3 hr, respectively, after injection. In tissues other than blood, concentrations were higher in liver and kidney than in spleen, heart, and lung.

Kellner, Christ, and Lötzsch (1973) followed the disappearance of ^{14}C-labeled benzidine and 3,3'-dichlorobenzidine in the blood of rats and dogs after single, intravenous injections of each compound at the rate of 0.2 mg/kg body weight. Half-lives of the labeled compounds in blood were: benzidine, 65 hr in the rat, 88 hr in the dog; dichlorobenzidine, 68 hr in the rat, 86 hr in the dog. As shown in Figures 6.1 and 6.2, blood concentrations of both compounds decreased relatively steadily, but the most rapid decreases occurred during the first 24 hr after injection.

In the same study, several measurements were made of the concentrations of the two compounds in various tissues of the dog, rat, and monkey.

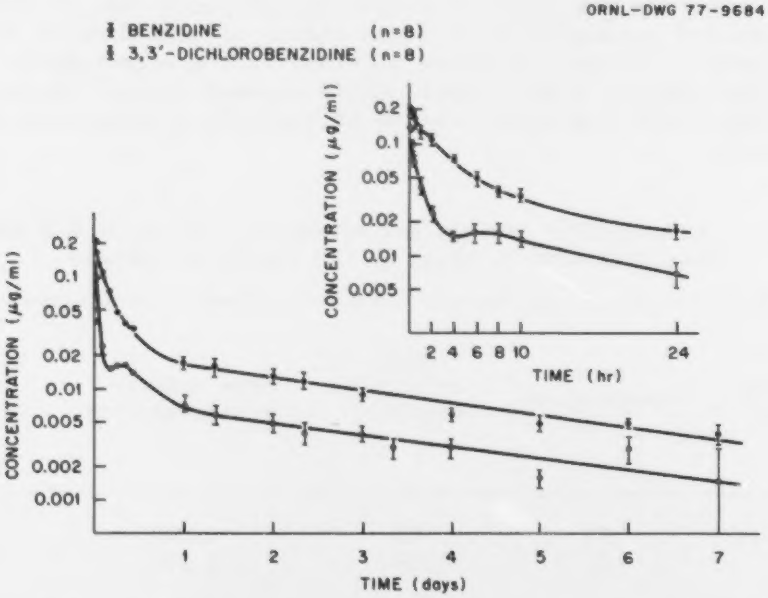

Figure 6.1. Concentration in blood after intravenous administration of 0.2 mg of ^{14}C-labeled 3,3'-dichlorobenzidine or benzidine per kilogram body weight to rats. Mean values and 95% confidence intervals. Source: Adapted from Kellner, Christ, and Lötzsch, 1973, Figure 1, p. 65. Reprinted by permission of the publisher.

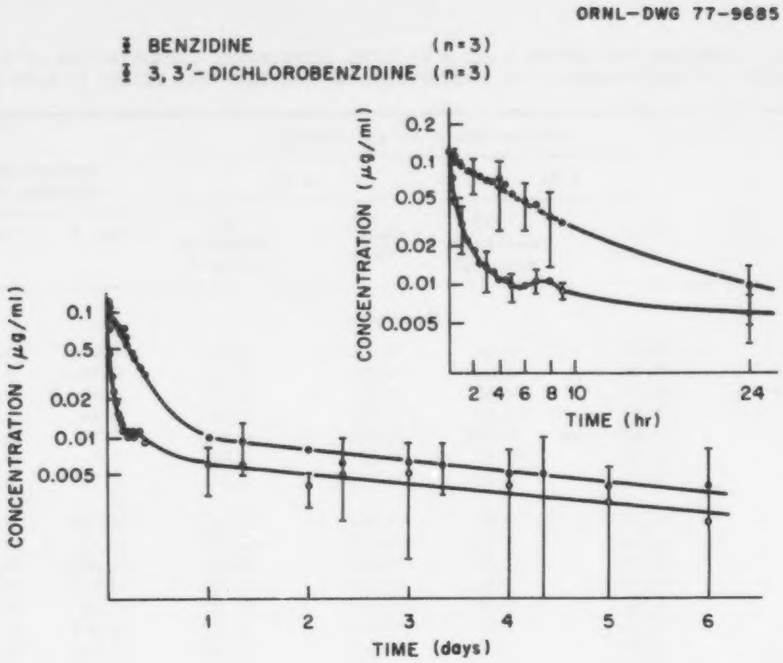

Figure 6.2. Concentration in blood after intravenous administration of 0.2 mg of ^{14}C-labeled 3,3'-dichlorobenzidine or benzidine per kilogram body weight to dogs. Mean values and 95% confidence intervals. Source: Adapted from Kellner, Christ, and Lötzsch, 1973, Figure 2, p. 65. Reprinted by permission of the publisher.

The results obtained are summarized in Tables 6.2 and 6.3. The only significant difference between the tissue concentrations of benzidine and dichlorobenzidine was in the urinary bladder wall of dogs at 4 hr after administration; this difference was thought to be caused by contamination of the wall with urine. At 4 hr after administration, concentrations of both compounds tended to be elevated in lung tissue of rats and in liver tissue of dogs. At 7 to 14 days after administration, elevated concentrations of benzidine were found in liver tissue of the rat, dog, and monkey and in lung tissue of the monkey. A somewhat similar pattern of distribution was found for dichlorobenzidine; elevated concentrations occurred in liver tissue of the rat, dog, and monkey and in lung tissue of the dog and monkey.

Baker and Deighton (1953) gave rats intraperitoneal injections of benzidine, 100 mg/kg body weight, and measured the concentrations found in various tissues and urine at 4 and 12 hr after injection. Concentrations, expressed as diazotizable amino groups, are given in Table 6.4. Highest concentrations of total diazotizable material were found in the stomach, stomach contents, and small intestine at 4 hr and in the small intestine and small intestine contents at 12 hr. Lowest concentrations

TABLE 6.2. DISTRIBUTION PATTERN 1 AND 4 hr AFTER INTRAVENOUS ADMINISTRATION OF 0.2 mg OF ^{14}C-LABELED 3,3'-DICHLOROBENZIDINE OR BENZIDINE PER KILOGRAM BODY WEIGHT TO RATS AND DOGS

Tissue	Concentration in rat tissues						Concentration in dog tissues, 4 hr (μg/g)		
	1 hr			4 hr					
	Mean value		95% confidence interval	Mean value		95% confidence interval	No. 4	No. 5	No. 6
				Benzidine					
Stomach	0.13		0.02	0.050		0.010	0.094	0.15	0.057
Small intestine	0.26		0.11	0.26		0.05	0.20	0.23	0.080
Large intestine	0.13		0.02	0.26		0.12	0.46	0.24	0.22
Kidney	0.27		0.03	0.13		0.03	0.24	0.32	0.21
Ureter	0.005	to	0.088	0.001	to	0.035	0.32	0.28	0.24
Urinary bladder	0.22		0.11	0.18		0.08	2.2	2.3	3.3
Liver	0.81		0.22	0.53		0.11	0.79	0.78	0.56
Bile							30	19	7.9
Pancreas	0.13		0.02	0.044		0.011	0.036	0.053	0.043
Spleen	0.14		0.02	0.067		0.017	0.12	0.16	0.11
Adrenals	0.24		0.05	0.086		0.024	0.081	0.096	0.080
Gonads, male	0.088		0.012	0.030		0.011	0.031	0.041	0.063
Lung	0.91		0.18	0.90		0.29	0.11	0.12	0.068
Heart	0.14		0.03	0.053		0.017	0.044	0.046	0.043
Skeletal muscle	0.093		0.019	0.030		0.006	0.030	0.026	0.054
Retroperitoneal fat	0.061		0.010	0.021		0.008	0.044	0.088	0.085
Subcutaneous fat	0.070		0.015	0.024		0.006	0.060	0.076	0.043
Brain	0.033		0.005	0.011		0.004	0.013	0.015	0.013
Mesentery lymph nodes	0.10		0.03	0.036		0.013	0.061	0.068	0.053
Blood	0.16		0.06	0.066		0.027	0.076	0.10	0.058
Plasma	0.22		0.07	0.092		0.031	0.12	0.16	0.094
				3,3'-Dichlorobenzidine					
Stomach	0.020		0.006	0.015		0.006	0.025	0.096	0.024
Small intestine	0.55		0.18	1.1		0.8	0.087	0.56	0.23
Large intestine	0.032		0.008	0.33		0.29	1.0	0.80	0.49
Kidney	0.096		0.024	0.078		0.011	0.078	0.046	0.041
Ureter	0.11		0.02	0.04		0.02	0.072	0.074	0.089
Urinary bladder	0.030		0.007	0.049		0.043	0.068	0.081	0.032
Liver	0.20		0.05	0.22		0.09	0.35	0.28	0.23
Bile							38	36	68
Pancreas	0.045		0.018	0.013		0.004	0.018	0.032	0.070
Spleen	0.024		0.005	0.012		0.002	0.013	0.022	0.014
Adrenals	0.24		0.13	0.086		0.037	0.050	0.032	0.045
Gonads, male	0.037		0.010	0.018		0.003	0.018	0.020	0.017
Lung	0.41		0.16	0.28		0.10	0.073	0.044	0.057
Heart	0.032		0.010	0.014		0.004	0.017	0.015	0.021
Skeletal muscle	0.016		0.002	0.007		0.003	0.017	0.016	0.013
Retroperitoneal fat	0.12		0.05	0.018		0.012	0.093	0.056	0.18
Subcutaneous fat	0.12		0.04	0.017		0.008	0.092	0.044	0.089
Brain	0.014		0.005	0.005		0.003		0.013	0.012
Mesentery lymph nodes	0.057		0.012	0.014		0.003	0.035	0.049	0.12
Blood	0.025		0.004	0.016		0.005	0.013	0.016	0.010
Plasma	0.041		0.007	0.028		0.008	0.026	0.031	0.019

Source: Adapted from Kellner, Christ, and Lötzsch, 1973, Table 4, pp. 75-76. Reprinted by permission of the publisher.

TABLE 6.3. DISTRIBUTION PATTERN 7 AND 14 DAYS AFTER INTRAVENOUS ADMINISTRATION OF 0.2 mg OF ^{14}C-LABELED 3,3'-DICHLOROBENZIDINE OR BENZIDINE PER KILOGRAM BODY WEIGHT TO RATS, DOGS, AND MONKEYS

Tissue	Concentration in wet tissues (μg/g)									
	Rat				Dog, 7 days			Monkey		
	7 days		14 days					7 days		14 days, No. 3
	Mean value	95% confidence interval	Mean value	95% confidence interval	No. 1	No. 2	No. 3	No. 1	No. 2	
				Benzidine						
Kidney	0.009	<0.001	0.006	<0.001	0.012	0.013	0.020	0.007	0.008	0.004
Ureter	0.001	<0.001	0.002	<0.001	0.001	0.002	0.003		0.001	0.002
Urinary bladder	0.005	<0.001	0.006	<0.001	0.007	0.007	0.007		0.003	0.002
Liver	0.042	0.002	0.022	0.004	0.12	0.19	0.087	0.027	0.010	0.011
Bile					0.086	0.13	0.13		0.007	0.004
Spleen	0.005	<0.001	0.010	0.001	0.029	0.029	0.030	0.003	0.002	0.003
Adrenals	0.004	<0.001	0.003	<0.001	0.016	0.010	0.016		0.002	0.003
Gonads, male	<0.001		0.001		0.002	0.002	0.008		0.005[a]	0.001
Lung	0.005	<0.001	0.005	<0.001	0.007	0.010	0.011	0.012	0.010	0.011
				3,3'-Dichlorobenzidine						
Kidney	0.006	<0.001	0.002	0.001	0.014	0.010	0.007	0.018	0.009	
Ureter					0.004	0.003	0.003	0.011	0.003	
Urinary bladder	0.004	<0.001	0.002	<0.001	0.005	0.003	0.003	0.015	0.018	
Liver	0.013	<0.001	0.004	<0.001	0.062	0.040	0.044	0.042	0.032	
Bile					0.60	0.18	0.13	0.055	0.019	
Spleen	0.004	<0.001	0.003	<0.001	0.004	0.005	0.004	0.012	0.006	
Adrenals	0.015	0.003	0.006	<0.001	0.004	0.005	0.005	0.016	0.004	
Gonads, male	0.002	<0.001	<0.001		0.002	0.002	0.002	0.006	0.001	
Lung	0.004	<0.001	0.002	<0.001	0.036	0.023	0.031	0.012	0.013	

[a] Female.

Source: Adapted from Kellner, Christ, and Lötzsch, 1973, Table 2, pp. 72-73. Reprinted by permission of the publisher.

TABLE 6.4. THE CONCENTRATION AND DISTRIBUTION OF FREE AND TOTAL DIAZOTIZABLE MATERIAL IN RAT TISSUES FOLLOWING A SINGLE INTRAPERITONEAL INJECTION OF 100 mg BENZIDINE PER KILOGRAM OF BODY WEIGHT

Tissue	Concentration after 4 hr				Concentration after 12 hr			
	Free		Total		Free		Total	
	(μg/g)	(% of total)	(μg/g)	(% of total)	(μg/g)	(% of total)	(μg/g)	(% of total)
Whole blood	16.2		35.0		<1.0		17.5	
Plasma	32.5	0.3	70.0	0.7	<1.0	<0.1	15.0	0.2
Blood cells	8.7	1.1	11.2	0.1	3.8	<0.1	5.0	0.1
Liver	16.2	1.7	45.0	4.8	11.3	1.6	25.0	3.5
Spleen	21.2	0.2	46.2	0.5	6.3	0.1	17.5	0.2
Kidney	21.2	0.2	46.2	0.5	2.5	0.1	11.2	0.2
Stomach	275.8	8.3	310.0	9.3	20.0	0.6	80.0	2.4
Stomach contents	310.0	15.6	315.0	15.8	54.0	2.7	85.0	4.3
Small intestine	81.2	13.0	157.[illegible]	25.2	26.3	2.2	135.0	11.5
Small intestine contents					134.0	6.7	560.0	28.0
Caecum	2.5	0.1	21.2	0.6	13.8	0.3	75.0	1.9
Caecum contents	2.0	0.1	11.4	0.6	0.1	<0.1	15.0	0.8
Colon	3.7	0.2	21.2	1.6	1.0	0.1	25.0	1.3
Urine	5.4	0.3	10.2	0.5	28.0	1.4	50.0	2.5
Miscellaneous[a]	1.7	0.3	21.2	3.8	1.0	0.2	11.2	2.0
Carcass	81.2	28.8	82.5	29.3	8.8	3.2	36.2	13.5
Total		69.2		93.3		19.20		72.4

[a] The miscellaneous sample consisted of brain, diaphragm, bladder, gonads, esophagus, heart, thymus, lungs, and adrenals.

Source: Adapted from Baker and Deighton, 1953, Table 1, p. 531. Reprinted by permission of the publisher.

were found in the red blood cells at both times. The measured, total diazotizable material accounted for 93% of the administered dose at 4 hr and 72% at 12 hr. Conjugated forms of the diazotizable material rose from 24% at 4 hr to 53% at 12 hr, indicating the formation of appreciable levels of metabolites. The content of total diazotizable material in urine was considerably higher at 12 hr than at 4 hr, with proportionately higher amounts of conjugated amines present. It was suggested that the difference between the amount of material injected and that measured during the 12-hr period was caused by in vivo oxidation to products not detectable by the method of measurement used. Since there is evidence that benzidine causes tumors in the liver of some animals, it may be of importance that measured concentrations in the liver were relatively high and remained fairly constant over the 12-hr period.

Pliss and Zabezhinsky (1970) reported on the distribution of free and bound amines in the tissues of rats given 20 mg of 3,3'-dimethylbenzidine subcutaneously once per week for eight weeks. Three days after the final injection the highest amine content was found in the Zymbal's gland with lesser amounts found in the kidney, omentum, spleen, and liver. A higher incidence of tumor development in the Zymbal's gland was observed in rats administered 3,3'-dimethylbenzidine.

Although no data that directly concern placental transfer of benzidine or its derivatives were found, studies made by Golub (1969) and Shabad et al. (1972) indicated transplacental effects of 3,3'-dichlorobenzidine and 3,3'-dimethylbenzidine on cultured embryonic kidney tissue. The tissue was obtained from pregnant mice treated with the benzidine derivatives at concentrations of 8 to 10 mg. Both derivatives promoted a longer survival rate of tissue and induced epithelial cell hyperplasia.

Although there is no evidence in the literature of any significant bioaccumulation of benzidine or its derivatives in human tissue or in tissues of the usual experimental animals, the *Federal Register* (1976) recently reported that edible portions of bluegill fish contained a 44-fold increase in residues of ^{14}C-benzidine over those present in water during a radiometric study.

6.2.3 Biotransformation and Elimination

Benzidine and its derivatives are wholly or partly transformed in humans and experimental animals to a number of metabolites, some of which are thought to act as proximate carcinogens in the induction of tumor formation. The metabolites, as well as the nontransformed parent compounds, are eliminated from the animal body via the urine and feces.

A summary of the biotransformation products of benzidine and its derivatives is provided in Table 6.5. The closely related compounds 4-aminobiphenyl and 4-nitrobiphenyl are included because of their structural and chemical similarities to benzidine and similar carcinogenic behavior in humans and animals. The specific chemistries of benzidine, its derivatives, and related compounds are covered in Section 2.2.

TABLE 6.5. METABOLITES FORMED BY BIOTRANSFORMATION OF BENZIDINE AND BENZIDINE DERIVATIVES IN ANIMALS

Compound	Species	Metabolite	Reference
Benzidine	Human	Acetyl N-hydroxy compound	Troll, Belman, and Rinde, 1963
	Human	N-Hydroxy acetylaminobenzidine	Haley, 1975
	Human	Monoacetylbenzidine and diacetylbenzidine	Haley, 1975
	Human	3-Hydroxybenzidine	Haley, 1975
	Human	3,3'-Dihydroxybenzidine	Haley, 1975
	Monkey	Monoacetylbenzidine	Rinde and Troll, 1975
	Dog	3-Hydroxybenzidine sulfate and glucuronide	Troll and Nelson, 1958
	Dog	3-Hydroxybenzidine hydrogen sulfate	Sciarini and Meigs, 1958
	Dog	3-Hydroxybenzidine	Bradshaw and Clayson, 1955 Sciarini, 1957
	Dog	4,4'-Diamino-3-diphenylyl hydrogen sulfate	Clayson, Ward, and Ward, 1959
	Dog	4-Amino-4-hydroxybiphenyl	Haley, 1975
	Dog	Monoacetylbenzidine and diacetylbenzidine	Haley, 1975
	Guinea pig	4'-Acetamido-4-aminodiphenyl	Clayson, Ward, and Ward, 1959
	Guinea pig	4'-Acetamido-4-amino-3-diphenylyl hydrogen sulfate	Clayson, Ward, and Ward, 1959
	Guinea pig	4'-Amino-4-diphenylyl sulfamic acid	Clayson, Ward, and Ward, 1959
	Guinea pig	N-Glucuronides	Clayson, Ward, and Ward, 1959
	Guinea pig	4'-Acetamido-4-diphenylyl sulfamic acid	Clayson, Ward, and Ward, 1959
	Rabbit	3-Hydroxybenzidine sulfate and glucuronide	Troll and Nelson, 1958
	Rabbit	4'-Acetamido-4-aminodiphenyl	Clayson, Ward, and Ward, 1959
	Rabbit	3-Hydroxybenzidine	Clayson, Ward, and Ward, 1959
	Rabbit	4'-Acetamido-4-amino-3-diphenylyl hydrogen sulfate	Clayson, Ward, and Ward, 1959
	Rabbit	4'-Amino-4-diphenylyl sulfamic acid	Clayson, Ward, and Ward, 1959
	Rabbit	4'-Acetamido-4-diphenylyl sulfamic acid	Clayson, Ward, and Ward, 1959
	Rabbit	N-Glucuronides	Clayson, Ward, and Ward, 1959
	Rat	3,3'-Dihydroxybenzidine	Haley, 1975
	Rat	N-Glucuronides	Elson, Goulden, and Warren, 1958 Clayson, Ward, and Ward, 1959
	Rat	4'-Acetamido-4-aminodiphenyl	Clayson, Ward, and Ward, 1959
	Rat	3-Hydroxybenzidine	Clayson, Ward, and Ward, 1959
	Rat	4,4'-Diamino-3-diphenylyl hydrogen sulfate	Clayson, Ward, and Ward, 1959
	Rat	4'-Acetamido-4-amino-3-diphenylyl hydrogen sulfate	Clayson, Ward, and Ward, 1959
	Rat	4'-Amino-4-diphenylyl sulfamic acid	Clayson, Ward, and Ward, 1959
	Rat	4'-Acetamido-4-diphenylyl sulfamic acid	Clayson, Ward, and Ward, 1959
	Mouse	Monoacetylbenzidine and diacetylbenzidine	Sciarini and Meigs, 1961*a*
	Mouse	Monoacetylated 3-hydroxybenzidine glucuronide and/or ethereal sulfate	Sciarini and Meigs, 1961*a*
	Mouse	N-Hydrogen sulfate and/or glucuronide	Sciarini and Meigs, 1961*a*
	Mouse	3-Hydroxybenzidine glucuronide	Sciarini and Meigs, 1961*a*
	Mouse	4'-Acetamido-4-aminodiphenyl	Clayson, Ward, and Ward, 1959
	Mouse	4,4'-Diamino-3-diphenylyl hydrogen sulfate	Clayson, Ward, and Ward, 1959
	Mouse	4'-Acetamido-4-amino-3-diphenylyl hydrogen sulfate	Clayson, Ward, and Ward, 1959
	Mouse	N-Glucuronides	Clayson, Ward, and Ward, 1959

(continued)

TABLE 6.5 (continued)

Compound	Species	Metabolite	Reference
3,3'-dimethylbenzidine (orthotolidine)	Human	Diacetyl-*o*-tolidine	Dieteren, 1966
	Human	5-Hydroxy-*o*-tolidine	Dieteren, 1966
	Human	Monoacetyl-*o*-tolidine	Dieteren, 1966
	Dog	5-Ethereal sulfate of *o*-tolidine	Sciarini and Meigs, 1961*b*
3,3'-Dimethoxybenzidine (dianisidine)	Dog	Unidentified diamine metabolite	Sciarini and Meigs, 1961*b*
3-Methoxybenzidine (mono-substituted dianisidine)	Rat	4-Amino-4'-acetamido-3-methoxybiphenyl	Laham, 1971
4-Aminobiphenyl	Monkey	N-Hydroxy-4-aminobiphenyl	Radomski et al., 1973
	Monkey	4-Nitrosobiphenyl	Radomski et al., 1973
	Monkey	N-Hydroxy-4-acetylaminobiphenyl glucuronide	Radomski et al., 1973
	Dog	4-Amino-3-diphenylyl hydrogen sulfate	Bradshaw and Clayson, 1955
	Dog	N-Hydroxy-4-aminobiphenyl	Radomski et al., 1973
	Dog	4-Nitrosobiphenyl	Radomski et al., 1973
	Dog	4-Amino-3-biphenylyl glucuronic acid	Gorrod, 1971, as cited in International Agency for Research on Cancer, 1972
4-Diphenylacetamide	Rat	N-Hydroxy-4-diphenylacetamide	Miller et al., 1961, as cited in International Agency for Research on Cancer, 1972
4-Nitrobiphenyl	Monkey	N-Hydroxy-4-aminobiphenyl	Radomski et al., 1973
	Monkey	4-Nitrosobiphenyl	Radomski et al., 1973
	Dog	N-Hydroxy-4-aminobiphenyl	Radomski et al., 1973
	Dog	4-Nitrosobiphenyl	Radomski et al., 1973

6.2.3.1 Human Studies — Englebertz and Babel (1953, as cited by Haley, 1975) identified free benzidine and its monoacetylated and diacetylated metabolites in the urine of a person receiving a single oral dose of 100 mg of benzidine. Not all of the dose received was recovered in the urine, indicating some additional fecal excretion or body accumulation. Troll, Belman, and Rinde (1963) identified free benzidine and N-hydroxy acetyl amino benzidine in the urine of persons receiving 200 mg of benzidine orally. In the analysis of urine from plant workers exposed to benzidine, Sciarini and Meigs (1961*a*) also found free benzidine and the monoacetylated and diacetylated derivatives and, in addition, found 3-hydroxybenzidine. The latter metabolite was the major excretion product, amounting to 78.5% to 89.7% of the total.

Total urinary excretion in the study by Sciarini and Meigs (1961*a*) was greater in August than in March, indicating greater uptake of benzidine by the workers during the warmer month of August. These results confirmed an earlier study made by Meigs, Brown, and Sciarini (1951) of plant workers exposed to benzidine. As shown in Figure 6.3, urinary excretion in this study was higher in June (mean for 12 men was 1.48 mg/liter) than in December (mean for 12 men was 0.433 mg/liter). Control urine from university or plant personnel entirely away from benzidine operations contained

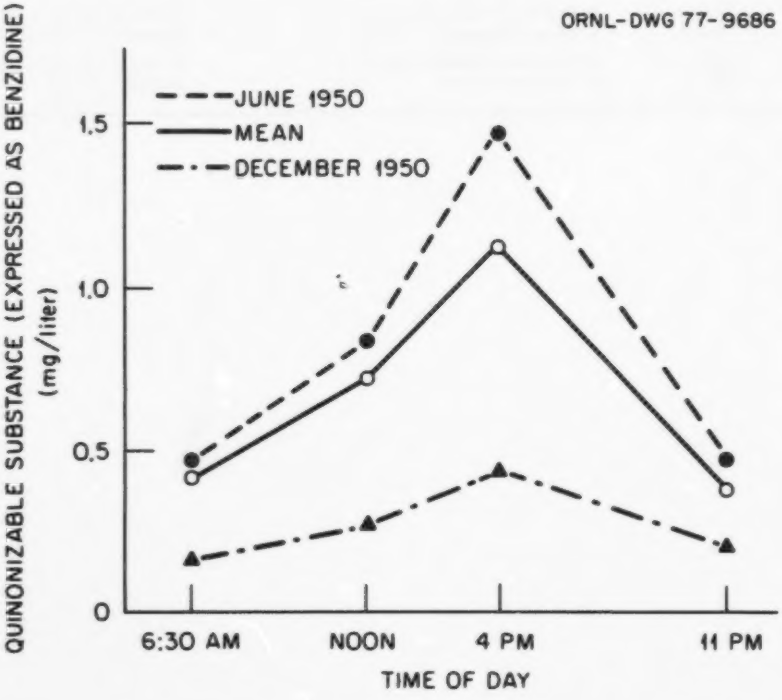

Figure 6.3. Trends of mean urinary concentration of quinonizable substance (expressed as benzidine) at different times of day over two-week periods in June and December 1950. Source: Adapted from Meigs, Brown, and Sciarini, Arch. Ind. Hyg. Occup. Med., Vol. 4, Figure 4, p. 539. Copyright 1951, American Medical Association.

no detectable benzidine. The higher urinary concentrations detected in June possibly were caused by greater concentrations of benzidine in the work area air. In a later study, Meigs, Sciarini, and Van Sandt (1954) suggested that air contaminated with 0.018 mg of benzidine compounds per cubic meter would lead, if inhaled for 8 hr, to a urinary concentration of not more than 0.026 mg/liter of diamines in urine voided at the end of a work shift. Hence, the authors concluded that a safe level of air exposure was probably 0.02 mg or less of benzidine per cubic meter of air.

Workers exposed to benzidine in a dyestuff factory were found by Vigliani and Barsotti (1962) to excrete, via the urine, benzidine and smaller amounts of 4-amino-4-oxybiphenyl and monoacetylbenzidine. No significant differences in urinary excretion were found between summer and winter periods of exposure.

Dieteren (1966) analyzed urine from chemical plant workers exposed to 3,3'-dimethylbenzidine (diorthotolidine) indicated the presence of free diorthotolidine, diacetyl-*o*-tolidine, and a hydroxyamino compound thought to be 5-hydroxy-*o*-tolidine. Although monoacetyl-*o*-tolidine could not be identified, there remained the possibility of its presence. These results indicate that in humans hydroxylation of diorthotolidine occurs in the *ortho* position with respect to the amino group and that, in this respect, the metabolisms of diorthotolidine and benzidine are similar.

Akiyama (1970) reported that workers handling benzidine yellow excreted 3,3'-dichlorobenzidine in their urine. This derivative of benzidine is part of the benzidine yellow molecule and presumably is liberated upon reductive metabolism of the dye.

6.2.3.2 Comparative Experimental Animal Studies — The biotransformation of benzidine to 3-hydroxybenzidine and to monoacetylated and diacetylated derivatives, as found in humans, also occurs in some experimental animals. Sciarini and Meigs (1961*a*) found that mice given benzidine intraperitoneally (100 mg/kg body weight) excreted in their urine the free benzidine, the monoacetylated and diacetylated metabolites, and, at somewhat higher concentrations, the ethereal sulfates and glucuronates of 3-hydroxybenzidine. Earlier, Sciarini (1957) found that dogs given benzidine intraperitoneally (100 mg/kg body weight) excreted free benzidine and conjugated 3-hydroxybenzidine. Sciarini and Meigs (1958) identified the conjugated metabolite as the ethereal sulfate of 3-hydroxybenzidine, which was recovered from the urine of dogs at rates of 25% to 50% of the intraperitoneal dose. Troll and Nelson (1958) reported that the major metabolites of benzidine in the dog and rabbit were the sulfate and glucuronide of 3-hydroxybenzidine. No unesterified hydroxy derivatives were found in dogs injected intraperitoneally with 1 g benzidine dihydrochloride or in rabbits given 100 to 300 mg of this compound. The metabolites were thought to result largely from *ortho* oxidation.

Differences appear among species of experimental animals in the metabolism and excretion of benzidine and its derivatives. Clayson, Ward, and Ward (1959) gave benzidine and 4'-acetamido-4-aminodiphenyl orally to dogs and rabbits and intraperitoneally to rats, mice, and guinea pigs. A summary of the metabolites produced by these species is given in Table 6.6.

TABLE 6.6. THE URINARY AND BILIARY METABOLITES OF BENZIDINE PRODUCED BY VARIOUS EXPERIMENTAL ANIMALS

Compound	Dog	Dog[a]	Rat	Mouse	Rabbit	Cavy
			Urinary			
Benzidine	+	+	+	+	±	±
4'-Acetamido-4-aminodiphenyl	0	±	+	±	±	±
3-Hydroxybenzidine (ether extracts)	+		+		±	
4,4'-Diamino-3-diphenylyl hydrogen sulfate	+	+	+	±	0	0
4'-Acetamido-4-amino-3-diphenylyl hydrogen sulfate	0	±	+	+	+	+
4'-Amino-4-diphenylyl sulfamic acid	0	0	±	0	+	±
4'-Acetamido-4-diphenylyl sulfamic acid	0	0	+	0	+	±
N-Glucuronides	+	+	+	±	+	+
Acid stable unknowns	1	1	3	3	3	0
			Biliary			
Benzidine	+		±		0	±
4'-Acetamido-4-aminodiphenyl	0		±		0	+
4,4'-Diamino-3-diphenylyl hydrogen sulfate	+		±		0	0
4'-Acetamido-4-amino-3-diphenylyl hydrogen sulfate	0		+		+	+
4'-Amino-4-diphenylyl sulfamic acid	0		+		0	0
4'-Acetamido-4-diphenylyl sulfamic acid	0		+		0	0
N-Glucuronides	+		±		+	+
Acid stable unknowns	1		3		0	0

[a] 4'-Acetamido-4-aminodiphenyl administered.

Source: Adapted from Clayson, Ward, and Ward, 1959, Tables I and II, pp. 582-583. Reprinted by permission of the publisher.

Dog urine contained free benzidine, 3-hydroxybenzidine, 4,4'-diamino-3-diphenylyl hydrogen sulfate, and N-glucuronides. Rat urine contained the same metabolites as well as 4'-acetamido-4-aminodiphenyl, 4'-acetamido-4-amino-3-diphenylyl hydrogen sulfate, 4'-amino-4-diphenylyl sulfamic acid, and three acid stable unknowns. Mouse urine contained only free benzidine and 4'-acetamido-4-amino-3-diphenylyl hydrogen sulfate. Rabbit urine contained the latter compound as well as 4'-amino-4-diphenylyl sulfamic acid, 4'-acetamido-4-diphenylyl sulfamic acid, and N-glucuronides. Guinea pig urine contained only 4'-acetamido-4-amino-3-diphenylyl hydrogen sulfate and N-glucuronides. These results suggest two major differences in benzidine metabolism among the species tested: (1) the dog was the only species tested unable to acetylate benzidine, and (2) the rabbit, rat, and guinea pig could convert benzidine to 4'-amino- and 4'-acetamido-4-diphenylyl sulfamic acid, whereas the dog and mouse could not.

Troll and Nelson (1958) found differences between dogs and rabbits in the excretion of amine metabolites of benzidine following intraperitoneal injection of benzidine. Dogs excreted 80% or more of the dose as compounds with an available aromatic amino group, whereas rabbits excreted only 50% of the dose as the same compounds. Also, dogs excreted relatively more of the sulfate conjugate and an unidentified amine fraction that was ether extractable.

Some differences in the amounts of excretion products found may result from varying periods of in vivo oxidation following administration. Thus, Baker and Deighton (1953) gave rats 100 mg benzidine per kilogram of body weight by intraperitoneal injection and, by analysis of the diazotizable material in tissues and urine 4 and 12 hr later, found that recovered amino groups rose from 24% to 49% in tissues over the 8-hr period and from 0.5% to 2.5% in urine over the same period. Sciarini and Meigs (1958) found that with decreasing dosage levels of benzidine given intraperitoneally to dogs the detection time in urine was comparatively decreased. A dose of 100 mg/kg body weight was detected in the urine for as long as seven days, whereas a dose of 20 μg/kg body weight was detected only in the first morning void.

Differences appear in the type and quantity of metabolites eliminated by different routes (i.e., urine, feces, and bile). In the work discussed above by Clayson, Ward, and Ward (1959), dog bile contained qualitatively the same metabolites as urine but in one-third the amount. Dog feces did not contain 3-hydroxybenzidine or the N-glucuronides present in the urine and bile.

Kellner, Christ, and Lötzsch (1973) determined that substitution of benzidine in the 3,3' positions affects the route of excretion in several animal species. Rats, dogs, and monkeys were injected intravenously with ^{14}C-labeled benzidine and 3,3'-dichlorobenzidine (2 mg/kg body weight). As shown in Table 6.7, differences in the main route of excretion were more apparent for benzidine than for dichlorobenzidine. In the rat, both benzidine and dichlorobenzidine were excreted in greater quantities in the feces than in the urine. In the dog, fecal excretion of dichlorobenzidine was again greater than urinary excretion, but fecal excretion of benzidine was much less than urinary excretion. In the monkey, neither route decisively excreted more of either compound, although there was a trend toward greater excretion of both compounds via the urine. Sciarini and Meigs (1961*b*) also found that dichlorobenzidine excretion by dogs was greater (by some ten times) in feces than in urine, and, in an earlier study (Sciarini and Meigs, 1958), they found just the opposite pattern for benzidine (i.e., some tenfold greater amounts excreted via the urine).

Biliary secretion is an important route of elimination. A portion of the products of benzidine metabolism in rats given intraperitoneal injections, 100 mg/kg body weight, was excreted by the bile to the stomach and intestinal tract (Baker and Deighton, 1953). As shown in Table 6.6, Clayson, Ward, and Ward (1959) detected some six metabolites of benzidine in bile of several animal species, but in only one-third the quantity found in urine. Kellner, Christ, and Lötzsch (1973) found benzidine and dichlorobenzidine in the bile of dogs and monkeys seven days after intravenous injection,

TABLE 6.7. EXCRETION IN THE FIRST SEVEN DAYS AFTER INTRAVENOUS ADMINISTRATION OF 0.2 mg OF ^{14}C-LABELED 3,3'-DICHLOROBENZIDINE OR BENZIDINE PER KILOGRAM BODY WEIGHT TO RATS, DOGS, AND MONKEYS

Species	Compound	Interval (days)	Amount excreted in urine (%)	t_{50} Phase IV (hr)	Amount excreted in feces (%)	t_{50} Phase IV (hr)	Amount in cage, feed (%)	Balance, 0 to 7 days (%)	Residues (%)
Rat	3,3'-Dichlorobenzidine	6	18 ± 4	49	79 ± 12	45	1	98 ± 12	2
	Benzidine	7	17 ± 2	38	80 ± 4	41	<1	97 ± 2	1
Dog	3,3'-Dichlorobenzidine	7	8 ± 6	37	84 ± 11		5	97 ± 8	3[a]
	Benzidine	7	66 ± 9	36	29 ± 13		1	96 ± 14	3[a]
Monkey 1	3,3'-Dichlorobenzidine	7	27		46		21	94	
Monkey 2	3,3'-Dichlorobenzidine	7	37		26		20	83	
Monkey 1	Benzidine	7	30		36		15	81	
Monkey 2	Benzidine	7	41		27		17	85	
Monkey 3	Benzidine	7	70		5		8	83	

[a] Content of liver, bile, and intestines.

Source: Adapted from Kellner, Christ, and Lötzsch, 1973, Table 1, p. 66. Reprinted by permission of the publisher.

but not in the bile of rats after an identical administration. However, the biliary concentrations found were low as compared with those found in urine or feces.

Rinde and Troll (1975) reported that the rhesus monkey can metabolically reduce a number of benzidine-based azo dyes to free the amine. They found substantial amounts of benzidine and monoacetylbenzidine in the urine of animals fed single doses of these dyes. This finding is in agreement with the finding by Akiyama (1970) (Section 6.2.3.1) that workers occupationally exposed to benzidine yellow (derived from 3,3'-dichlorobenzidine) excreted dichlorobenzidine in their urine, presumably through metabolic reduction of this dye.

The metabolism and excretion of benzidine derivatives and related compounds have been less extensively studied than that of benzidine. Sciarini and Meigs (1961*b*) injected dogs intraperitoneally with 3,3'-dimethylbenzidine (diorthotolidine), 3,3'-dimethoxybenzidine (dianisidine), and 3,3'-dichlorobenzidine. Administered levels of these compounds were only partly accounted for by measured, urinary excretory products. All three diamines were recovered partly in their nonmetabolized form. A metabolite of diorthotolidine, thought to be the 5-ethereal sulfate, was recovered at concentrations ranging from 15% to 40% of the administered dose. A metabolite of dianisidine was also recovered, but at a relatively lower percentage yield. However, no metabolite of dichlorobenzidine was found. Laham (1971) detected an acetyl derivative, 4-amino-4'-acetamido-3-methoxybiphenyl, in the urine of rats following injection of 3-methoxybenzidine, the mono-substitute of dianisidine. Radomski et al. (1973) found 4-nitrosobiphenyl and N-hydroxy-4-aminobiphenyl in the urine of dogs and rhesus monkeys fed 5 mg of 4-aminobiphenyl per kilogram body weight. Monkey urine contained a third metabolite, N-hydroxy-4-acetylaminobiphenyl glucuronide. Urinary excretion of the N-hydroxy metabolites was more rapid in both animals than that of the administered 4-aminobiphenyl, with the maximum excretion occurring at 4 to 6 hr (Figure 6.4). Bradshaw and Clayson (1955) fed dogs 200 mg/day of 4-aminobiphenyl and recovered the parent compound and a metabolite, 3-hydroxy-4-aminobiphenyl from the urine. Bradshaw (1959) calculated recoveries in the urine of several species of animals fed 4-aminobiphenyl. The yield of the 3-sulfuric acid conjugate measured in the urine was 25% to 40% in dogs, 3% to 7% in rats, 1% to 2% in hamsters, 1% in mice, and 0% in guinea pigs.

6.3 EFFECTS

As noted by Clayson (1976), there is convincing evidence that benzidine is a bladder carcinogen in humans and that benzidine and several derivatives are carcinogenic in experimental animals. In addition to their carcinogenic potential, there is also evidence that these compounds cause dermatitis, cystitis, and hematuria in humans and a number of additional effects in experimental animals. A summary of these effects is provided in Table 6.8.

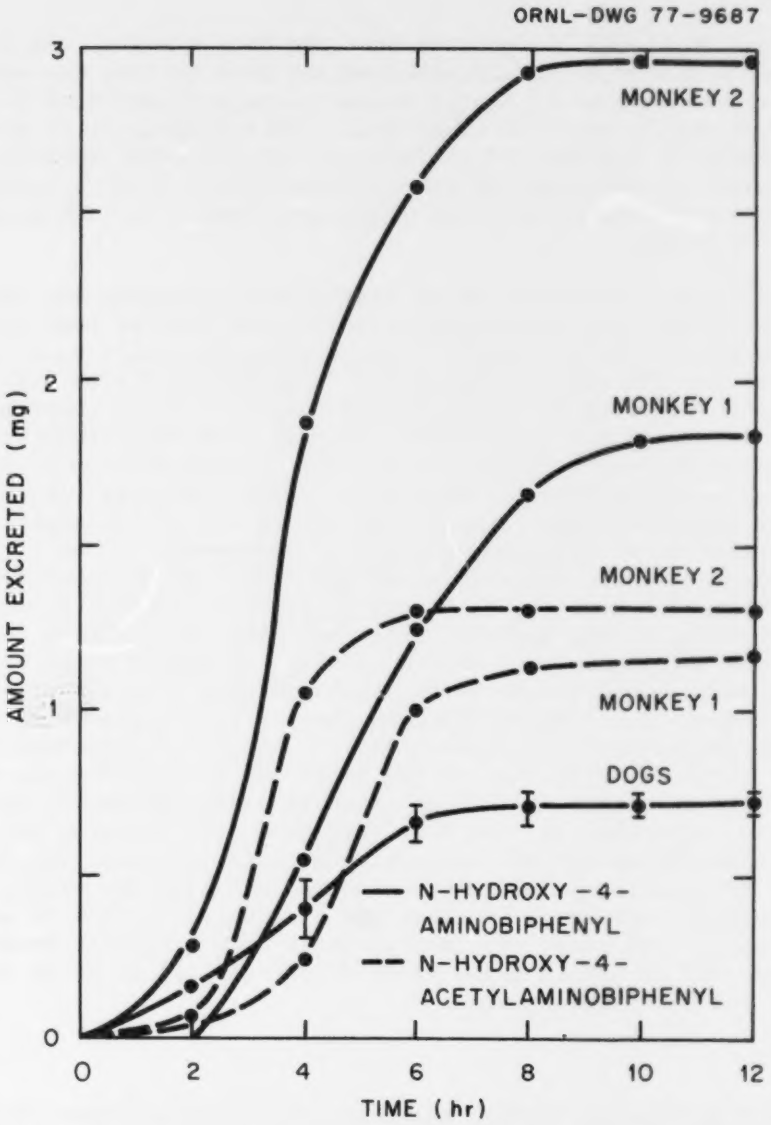

Figure 6.4. Comparative excretion of N-oxidation products of 4-aminobiphenyl by monkeys and dogs. Source: Adapted from Radomski et al., 1973, Figure 2, p. 992.

TABLE 6.8. EFFECTS OF BENZIDINE, ITS CONGENERS, AND METABOLITES ON VARIOUS ANIMAL SPECIES

Species	Carcinogen	Effect
Mouse	Benzidine	Hepatoma, lymphoma, bile duct proliferation
	3,3'-Dihydroxybenzidine	Hepatoma, lymphoma, bile duct proliferation, benign bladder papilloma
Rat	Benzidine and its sulfate	Cirrhosis of liver, hepatomas, carcinoma of Zymbal's gland, adenocarcinoma, degeneration of bile ducts, sarcoma, mammary gland carcinoma
	3,3'-Dihydroxybenzidine	Hepatoma, adenocarcinoma of colon, carcinoma of forestomach, Zymbal's gland carcinoma, bladder carcinoma
	Dianisidine[a]	Zymbal's gland carcinoma, ovarian tumor
	o-Ditoluidine[b]	Papilloma of stomach, Zymbal's gland carcinoma, mammary tumor, leukemia
	3,3'-Benzininedioxyacetic	Papilloma of bladder, hepatic sarcoma
	3,3'-Dichlorobenzidine	Extensive cancer
	N,N'-Diacetylbenzidine	Chronic glomerulonephritis
Hamster	Benzidine	Hepatoma, liver carcinoma, cholangiomas
	o-Ditoluidine[b]	Bladder cancer
Rabbit	Benzidine	Proteinuria, hematuria, liver cirrhosis, myocardial atrophy, bladder tumor, gall bladder tumor
Dog	Benzidine	Recurrent cystitis, bladder tumor, convulsions, liver cirrhosis, hematuria
Monkey	Benzidine	No pathological changes
Human	Benzidine	Bladder tumor, papilloma, chronic cystitis, hematuria

[a] 3,3'-Dimethoxybenzidine.
[b] 3,3'-Dimethylbenzidine.

Source: Adapted from Haley, 1975, Table 5, p. 24. Reprinted by permission of the publisher.

6.3.1 Physiological Effects

During in vitro studies on benzidine, its derivatives, and related compounds, Hirai and Yasuhira (1972) found a possible relationship between the potential carcinogenicity of these compounds and their ability to reduce cytochrome *c*. 3,3'-Dichlorobenzidine and *p*-aminobiphenyl failed to reduce cytochrome *c*; benzidine, 3,3'-dimethylbenzidine, and 3,3'-dimethoxybenzidine were moderate reductants; and 3,3'-diaminobenzidine was a strong reductant. Cammer and Moore (1973) also reported that 3,3'-diaminobenzidine markedly reduced cytochrome *c*. Thus, a compound such as *p*-aminobiphenyl, considered to be strongly carcinogenic, displayed weak reducing power, whereas 3,3'-diaminobenzidine, considered to be weakly carcinogenic, displayed strong reducing power. The authors suggested that aminobiphenyls which are easily oxidizable by mitochondrial systems may be detoxified in this manner and excreted in combination with sulfuric and amino acids.

Exposure to benzidine alters the activity of several enzymes. The activity of β-glucuronidase in urine of exposed workers is significantly increased when compared with activity levels in healthy unexposed workers (Kleinbauer et al., 1969; Popler, Selucky, and Vlasak, 1964). Removal of workers from benzidine exposure decreased the enzyme activity; however, the decrease did not reach the level in unexposed workers.

Exposure to benzidine also decreases enzyme activity. Subcutaneous administration of benzidine to rabbits caused a decrease in blood phenolase activity (Nakajima, 1955), whereas diorthotolidine did not cause this decrease. Since blood phenolase activity decreases prior to the appearance of anemia, phenolase activity may be a means of early diagnosis of poisoning by aromatic derivatives. Catalase and peroxidase activity was decreased in rats injected with benzidine (Soloimskaya, 1968). Along with the decreased enzyme activity, there was a decrease in erythrocytes and thrombocytes and an increase in leucocytes.

Along with decreasing enzyme activity in rats, an increase in male rat liver glutathione occurred with exposure to benzidine (Neish, 1967). The compound was injected intraperitoneally at a dose of 12.7 mg/100 g body weight. The control glutathione value was 182 mg %, whereas the value 24 hr following benzidine injection was 272 mg %.

6.3.2 Toxicity

6.3.2.1 General Toxicity — Benzidine and benzidine derivatives cause poisoning in laboratory animals resulting in glomerulonephritis and nephrotic syndrome. Indirect evidence of transplacental effects on embryonic tissues also has been observed. In humans, benzidine compounds have been associated with dermatitis, cystitis, and hematuria.

6.3.2.1.1 Dermatitis — Schwartz, Tulipan, and Peck (1947) reported cases of dermatitis among workers in several dye manufacturing plants and attributed a portion of these cases to benzidine. Both benzidine and 3,3'-dimethylbenzidine have caused dermatitis in chemists who work in laboratories. Various benzidine color dyes have been implicated as causing dermatitis; however, not all exposed workers are irritated by these compounds. Individual sensitivity plays a role in determining dermatitis development.

6.3.2.1.2 Glomerulonephritis and nephrotic syndrome — N,N'-Diacetylbenzidine has been used to produce a nephrotic syndrome in rats which morphologically resembles a rapidly progressive human glomerulonephritis (Harman et al., 1952; Harman, 1971). Both sexes of Sprague-Dawley rats were fed a grain diet containing 0.043% N,N'-diacetylbenzidine. Proteinuria developed in both sexes within three to four weeks. In females, proteinuria rapidly became severe until as much as 0.1 g of protein was excreted within a 24-hr period following two months on the diet. Severe anemia in females appeared following proteinuria (Table 6.9), whereas in the male rats anemia was rare and never severe. Females, after developing proteinuria, displayed hypoproteinemia, hyperlipemia, generalized edema, and, as previously mentioned, anemia. Glomerular lesions which consisted of florid epithelial crescents, progressive sclerosis, and obliteration of many glomeruli appeared in females. Similar renal lesions developed in males; however, their appearance was slower and less extensive. Only a mild nephrotic syndrome was found in male rats. Atrophy of the testes developed in all males which showed the syndrome. Morphological similarities between the nephrotic syndrome produced in rats by N,N'-diacetylbenzidine and the human syndrome included extracapillary cell proliferation, formation of luxuriant crescents in 80% of the glomeruli, intact tufts in almost all glomeruli, and the prevalence of many normal glomeruli in advanced grades of the syndrome.

Rats fed N,N'-diacetylbenzidine and 4,4,4',4'-tetramethylbenzidine at equal molar levels of 0.25% and 0.27%, respectively, developed lipemia and glomerular lesions consisting of fat-filled spaces in the glomerular tuft from 2 to 4.5 months after beginning of treatment (Dunn, Morris, and Wagner, 1956). No difference in the effects of the two compounds was observed. Two groups of inbred mice fed these compounds at the same concentration showed no effects. N,N'-Diacetylbenzidine introduced by subcutaneous (100 mg) or intraperitoneal (100 or 200 mg) injection produced severe glomerulonephritis in 5 rats and mild lesions in 12 rats (Bremner and Tange, 1966). Renal lesion severity may have varied with the dose of

TABLE 6.9. DEVELOPMENT OF PROTEINURIA AND ANEMIA IN FOUR FEMALE RATS FED A DIET CONTAINING N,N'-DIACETYLBENZIDINE

Observation	Mean value after feeding			
	0-3 weeks	4-8 weeks	9-10 weeks	11 weeks
Red cell count, $10^6/\mu l$	8.23	7.69	6.81	3.51
Daily protein loss in urine, mg	16.54	378.67	314.38	250.63

Source: Adapted from Harman, 1971, Table III, p. 123. Reprinted by permission of the publisher.

the compound. In another study, Christopher and Jairam (1970) reported a low-grade type of glomerulonephritis present in white rats fed 4,4'-diaminobiphenyl.

6.3.2.1.3 Subacute toxicity — Mice daily fed 0.01% to 0.08% benzidine dihydrochloride exhibited various toxic symptoms (Rao et al., 1971). The mice lost weight in proportion to the dose of benzidine received, with males losing up to 20% and females up to 7% of their body weight. The weights of the carcass, liver, and kidney decreased with an increase in dose, whereas the spleen and thymus weights increased with dose increases. Among morphological effects were cloudy swelling of the liver, vacuolar degeneration of renal tubules, and hyperplasia of the myeloid elements in bone marrow and of lymphoid cells in the spleen and thymic cortex. Male mice appeared more sensitive to benzidine than did female mice. This finding seems to be in disagreement with that of Harman (1971), who reported female rats more sensitive than male rats. However, this difference may be a species difference, a difference in benzidine compounds, or a combination of these two possibilities.

6.3.2.1.4 Indirect evidence of transplacental effects — Golub (1969) and Shabad et al. (1972) found that 3,3'-dichlorobenzidine and 3,3'-dimethylbenzidine increased survival time of embryonic mouse kidney tissue cultures and produced hyperplastic changes in the epithelium. Eight to 10 mg of either compound injected subcutaneously into pregnant mice produced hyperplastic growth that was nodular or diffuse or that formed solid, compact areas in explanted embryonic kidney tissue five to six days after administration (Table 6.10). Longer survival of the explanted kidneys was promoted by treatment with these two compounds during pregnancy. Administration of a total dose of 8 to 10 mg per mouse of 3,3'-dimethylbenzidine by subcutaneous injection to BALB/c mice during the last week of pregnancy produced an increased incidence of tumors in the progeny (Golub, Kolesnichenko, and Shabad, 1974). Eight of 16 mice had tumors; 6 with adenomas of the lungs and 5 with tumors of the mammary gland. The tumors found in the progeny could have been induced by transplacental action or by transmission of 3,3'-dimethylbenzidine through milk of the lactating mothers.

6.3.2.2 Carcinogenicity — Benzidine and several other aromatic amines have been implicated as carcinogens of the urogenous organs, particularly the bladder (Hueper, 1954). However, it is generally accepted that metabolites of these aromatic amines, referred to as proximate carcinogens, are the active compounds (Clayson, 1969). The metabolites are formed by conversion of the aromatic amines to ring hydroxylated compounds which then are N-hydroxylated, acylated and deacylated, and conjugated with sulfate and glucuronic acid (Haley, 1975). Evidence suggests that the N-hydroxylation produces the precarcinogen and that the sulfate or glucuronidate conjugates are the carcinogenically active forms in vivo. Metabolites produced by experimental animals apparently have an affinity to and exert a carcinogenic action on organs other than the bladder (Hueper, 1961). The carcinogen apparently must be administered to the animal a considerable time before tumors are produced.

There is strong experimental evidence that benzidine and related compounds induce various tumors in laboratory animals. As noted in Table 6.8,

TABLE 6.10. SURVIVAL AND HYPERPLASTIC CHANGES OF EPITHELIUM IN EMBRYONIC KIDNEY TISSUE CULTURES TREATED TRANSPLACENTALLY WITH 3,3'-DICHLOROBENZIDINE AND 3,3'-DIMETHYLBENZIDINE

Duration of experiment (days)	3,3'-Dichlorobenzidine-treated culture			3,3'-Dimethylbenzidine-treated culture		
	Number of surviving explants in control cultures/total[a]	Number of surviving explants/total	Hyperplastic changes (outgrowths)	Number of surviving explants/total	Hyperplastic changes	
					Hyperplasia of tubules	Outgrowths
3-4	13/13	3/3	2	6/6	2	1
6-7	12/12	6/6	15	8/8	1	1
14-15	0/23	48/56	3	12/16		4
18-20	0/7	9/13				
Total	25/55	68/78	20	26/30	3	6
Total survival, %	45.5	84.9		86.9		
Total percentage of hyperplastic growth			30.4		11.5	23.1

[a] Control cultures did not reveal any hyperplastic changes.

Source: Adapted from Shabad et al., 1972, Table 6, p. 621. Reprinted by permission of the publisher.

many of these are located in the liver. In single experiments without confirmation, bladder cancer induction in experimental animals was limited to the dog and rabbit.

As pointed out by Haley (1975), a serious problem exists with the animal experimentation so far accomplished in that experiments have always been carried out at toxic doses that resulted in liver failure and death of the majority of the animals prior to tumor development. He suggested that one means of correcting this problem would be the performance of a 90-day subacute study in several species at dosage levels below those known to produce liver pathology.

Despite the substantial evidence accumulated indicating a strong association between occupational exposure to benzidine and the development of bladder cancer in humans, a cause-effect relationship has not been established in the epidemiological sense. (See MacMahon and Pugh, 1970, for epidemiological procedure and meaning.) A note of caution, therefore, seems warranted. In the following discussion of a number of studies made on occupationally exposed workers, the proportions of persons with cancer are noted for specific periods. Such proportional rates do not express the real magnitude of the risk associated with the exposures encountered. However, this magnitude can be determined by comparing the rate of bladder cancer among the exposed workers with the rate of bladder cancer among the unexposed population at large. This concept of risk is useful in identifying high-risk groups. For example, Case et al. (1954) reported that the risk of developing bladder cancer was 14 times greater among workers exposed to benzidine than in persons without such exposure.

6.3.2.2.1 Benzidine — Benzidine has been associated with a high occurrence of bladder cancer in exposed industrial workers (International Agency for Research on Cancer, 1972; Riches, 1972; Sax, 1975). Malignancies have been reported following only a few weeks of exposure and a latent period of several years (Deichmann and Gerarde, 1969). Hamblin (1963) reported an average latent period of 18.6 years for bladder tumors. The development of tumors from benzidine exposure seems to be related to the degree of control of exposure afforded by work habit and personal hygiene programs (Rye et al., 1970). The occurrence of tumor formation is affected by initial exposure concentration, exposure duration, and years of survival following exposure. However, there is a lack of data on the benzidine concentrations to which workers have been exposed.

Zavon, Hoegg, and Bingham (1973) reported 13 of 25 exposed men developed tumors of the bladder. This unusually high occurrence rate may have been due to the long exposure period. The workers were exposed to other chemicals; however, benzidine was the chemical common to all and was indicated as the carcinogen. Those who developed tumors were exposed to benzidine more than 13 years, whereas the nontumor group was exposed less than 9 years. The tumor recurrence rate was 62% (Wendel, Hoegg, and Zavon, 1974). Samples of benzidine atmospheric concentrations taken from various locations in the benzidine manufacturing plant ranged from less than 0.005 to 0.415 mg/m^3, except in one location where the concentration was 17.6 mg/m^3.

Bladder tumors in benzidine-exposed workers are found in all countries in which this chemical is manufactured and used. In France, 85 cases of bladder tumors in men working in aromatic amine manufacturing plants were found before 1960 (Billiard-Duchesne, 1960). Fifty-one cases originated in a factory in Normandy; 17 cases from this factory occurred prior to 1947 and 34 subsequent to 1947. Eighteen of those 34 had symptoms of hematuria and stranguria. Seven of the 18 were not directly exposed to the amines, but they had worked in the factory an average of 25 years. Kuzelova, Kunor, and Hurt (1969) reported positive findings in 17% of those examined for urinary system tumors in workers employed in benzidine production. The average length of exposure was eight years; the highest rate of positive findings was among those with six to ten years of exposure.

Gehrman (1936) reported occurrence of bladder tumors among workers in the American dye industry. A total of 24 cases of carcinoma appeared in workers exposed to aromatic amines, including benzidine. The average exposure time necessary to produce tumors was 12 years. Case et al. (1954) found that workmen exposed to benzidine in the dyestuff industry in England had more bladder tumors than unexposed workers. The induction period averaged 16 years; however, some tumors appeared in less than 2 years. Reporting on the occurrence of bladder tumors in a British dyestuff factory, Scott (1952) found 66 cases of bladder tumors in which 30 were of workers exposed to benzidine. The latent period averaged 15.9 years and ranged from 8 to 32 years (Figure 6.5). The intensity of exposure in the benzidine manufacturing was thought to be a factor in producing earlier tumors among these workers than among workers engaged in benzidine handling. Of 23 cases of bladder tumors in benzidine-exposed workers in manufacturing, 14 had papilloma, 7 had carcinoma, and 2 had papilloma which recurred as carcinoma.

Studies on the occurrence of bladder tumors among workers in Italian dyestuff factories indicated a higher proportion among benzidine-exposed workers than among unexposed workers. Vigliani and Barsotti (1962) found 24 cases of bladder tumors in a total of 109 workers exposed to benzidine or benzidine and β-naphthylamine. From 1931 to 1960, 47 bladder tumors were found in workers exposed to benzidine. Of these, 21 were carcinomas and 16 were papillomas. From 1931 to 1948, 13 cases of carcinoma of the bladder were detected in 83 workers exposed to benzidine in a dyestuff plant (Barsotti and Vigliani, 1952). Men working in filtration, pressing, drying, and milling in the benzidine processing had the greatest exposure. Workers with tumors had been exposed to benzidine from 5 to 16 years with an average exposure of 8 years. The maximum time between cessation of benzidine contact and tumor development was 16 years. Over a period of six years (1964 to 1970), Forni, Ghetti, and Armeli (1972) examined 858 workers in five dyestuff factories and found 17 cases of bladder tumor. Of these, ten were papillomas and seven were carcinomas.

Tsuchiya, Okubo, and Ishizu (1975) studied 100 cases of occupational bladder cancer reported from 1949 to 1970 by dye-producing companies in Japan. Only a few of these cases were reported prior to 1955 because of the long latent period involved. The proportion of bladder tumors was 11.25% among workers involved in benzidine production and 1.45% among

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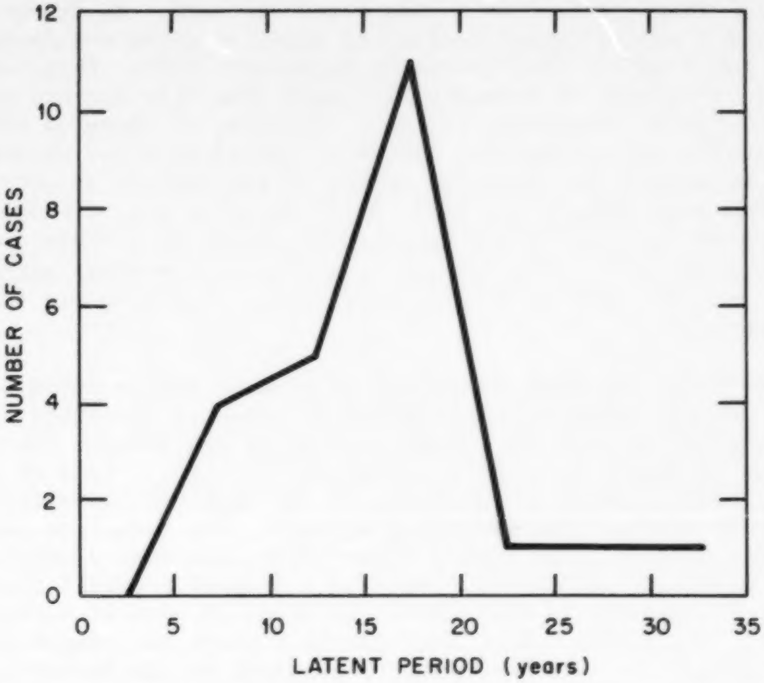

Figure 6.5. Graph of time from initial exposure to benzidine to diagnosis of tumors. Source: Adapted from Scott, 1952, Figure 3, p. 129. Reprinted by permission of the publisher.

those concerned with benzidine use. Eight of the 100 cases developed tumors in the upper urinary tract that did not involve the bladder. The average latent period for those exposed to benzidine was 16.25 years with a standard deviation of 7.38 years. A high risk of bladder tumors among silk kimono painters in Kyoto was reported by Yoshida and Miyakawa (1973). These painters moistened brushes with the tongue and accidentally ingested benzidine dyes, probably causing the bladder tumors.

Workers in the coal tar dye industry also have a high occurrence of bladder tumors. Goldwater, Rosso, and Kleinfeld (1965) reported 21.3% of employees exposed to benzidine contracted malignant bladder tumors in a coal tar dye plant. Those exposed to benzidine plus β-naphthylamine had a higher rate of 45.5%. The average latent period until tumor development was 18.4 years for papillomas and 18.7 years for malignancies. In a later report, Kleinfeld, Rosso, and Goldwater (1966) also reported a higher proportion of bladder tumors in coal tar dye plant workers exposed to benzidine plus β-naphthylamine than among workers exposed to only benzidine.

Mancuso and El-Attar (1966, 1967) found a higher proportion of bladder and kidney cancer in benzidine-exposed workers in a plant manufacturing benzidine and β-naphthylamine than that found among unexposed workers.

Of 171 deaths among the exposed workers, 18 were reportedly due to bladder and kidney cancers. There was also a higher rate of malignant neoplasms of the digestive system. Exposure to benzidine plus β-naphthylamine was apparently more potent than exposure to benzidine alone.

In a report of carcinogenic hazards in microbiology laboratories, Wood and Spencer (1972) suggested that benzidine exposure in the laboratory could cause bladder cancer. Benzidine is used in clinical biochemical laboratories and presents a carcinogenic hazard to technicians (Collier, 1974; Veys, 1972). Congo Red, a benzidine-based azo dye, has been used as a bacteriostatic agent in the isolation of tubercle bacilli from sputum (Frobisher, 1946) and is still employed as an indicator dye in the mixed culture of *Rhizobium* species and related species of bacteria (Vincent, 1970, pp. 4-5).

Occupational tumors of the bladder from benzidine exposure have a long latent period averaging 18 years. Age is not considered a factor in disease development (Hamilton and Hardy, 1974). These occupational bladder tumors appear to be similar to bladder tumors found in the general public and have a tendency for high recurrence.

Benzidine is carcinogenic in several animal species under varying conditions (Casarett and Doull, 1975). There appears to be, however, some controversy about the induction of bladder tumors by benzidine in dogs. Haley (1975) stated that benzidine does in fact produce bladder tumors in dogs. Spitz, Maguigan, and Dobriner (1950) found a papillary carcinoma of the urinary bladder in one of seven dogs fed benzidine for five years. The tumor was found seven and one-half years after the start of the experiment. Benzidine administered orally to dogs has not produced bladder cancer (Marhold et al., 1968). Deichman et al. (1965) fed female beagle dogs 1 mg/kg of benzidine per dog five times a week for three years; no tumors were found. The carcinogenic actions of 2-naphthylamine, benzidine, 4-aminobiphenyl, and 4-nitrobiphenyl were not additive when the compounds were fed together.

Saffiotti et al. (1967) failed to induce bladder tumors in 30 male and female Syrian golden hamsters fed benzidine at 0.1% of the diet throughout their life spans. Carcinogenic effects were produced in other organs; extensive bile duct proliferations and cysts appeared, with cholangiofibrosis and liver cell carcinoma and hepatomas.

Benzidine injected subcutaneously into rats at 15 mg/week produced hepatic injury followed by cirrhosis (Spitz, Maguigan, and Dobriner, 1950). Sebaceous gland carcinomas were found as well as hepatomas and adenocarcinomas of the rectum. No bladder tumors were produced. Baker (1950) subcutaneously injected Albino Delph mice with 300 mg benzidine or 300 mg dihydroxybenzidine, a suspected benzidine metabolite produced in humans. Both bladder and liver changes were found, including benign papillomas of the bladder. Table 6.11 summarizes these effects. Dihydroxybenzidine produced benign papillomas in the bladders of the mice, whereas mice receiving benzidine did not show these effects. Later, Baker (1953) found changes in the liver, including cirrhosis and hepatomas, adenocarcinoma

TABLE 6.11. BLADDER AND LIVER CHANGES PRODUCED IN ALBINO DELPH MICE ADMINISTERED BENZIDINE OR DIHYDROXYBENZIDINE BY SUBCUTANEOUS INJECTION

Group	Chemical administered	Number surviving 45 weeks or more	Normal bladder	Hyperplasia	Papillomas		Normal liver	Bile duct proliferation	Hepatomas	
					Benign	Malignant			Benign	Malignant
A1	300 mg benzidine base	9	9				1	3	4	1
A2	300 mg 3,3'-dihydroxy-4,4'-diaminodiphenyl	9	2	1	5		5	1	3	
B1, B2	Olive oil	19	17	2			13	3	3	
C1, C2	Untreated	17	17				12		3	2 ?

Source: Adapted from Baker, 1950, Tables II and III, p. 49. Reprinted by permission of the publisher.

of the colon, carcinoma of the sebaceous gland next to the external auditory canal (Zymbal's gland), and squamous-cell carcinoma of the stomach in rats fed 5 to 8 g/day of dihydroxybenzidine (0.125% of a normal basic diet). In three of the seven surviving animals, one sessile papilloma and two keratinized squamous-cell carcinomas of the bladder wall appeared. Wistar rats injected intraperitoneally or subcutaneously with 100 or 200 mg of N,N'-diacetylbenzidine developed tumors primarily in the external auditory canals and mammary glands 6 to 15 months following injection. Twenty-four tumors were formed in 14 of 30 rats. Severe glomerulonephritis was found in 5, and mild lesions were found in 12 animals. Renal lesion severity probably varied in relation to the size of the dose. Griswold et al. (1968) also reported carcinomas of the mammary gland in female Sprague-Dawley rats given 12 to 50 mg of benzidine per rat orally. One fibroadenoma was also found in the mammary gland. However, Marhold et al. (1967, 1968) found no tumors in Wistar rats fed 2 mg of benzidine per rat per day for their life spans.

Rats given benzidine (the amount of dose was not specified) by subcutaneous injection for six months developed cirrhosis early in the study (Pliss, 1963). Hepatomas, tumors of the Zymbal's gland, and sarcomas at the injection site were found. These tumors made up 70% of those found in the rats (Pliss, 1964). Benzidine was more toxic to female rats than to male rats. Injection of 3,3'-benzidine dicarboxylic acid in mice and rats over a period of a year produced tumors of the Zymbal's gland and the liver (Pliss, 1969). Four of 28 rats injected weekly with 5 mg benzidine developed intestinal tumors, and 4 of 37 rats injected weekly with 5 mg benzidine plus 10 mg orthotolidine had intestinal tumors (Pliss, Volfson, and Jogannsen, 1973). The activity of benzidine plus orthotolidine did not appear to be additive.

Of 22 rats given an accumulative dose of 0.75 g/kg body weight of benzidine for 15 days, 20 rats developed tumors, including 19 hepatomas, 18 cholangiomas, 7 intestinal tumors, and 4 carcinomas of the sebaceous gland (Holland et al., 1974). Tetramethylbenzidine (4.15 to 8.3 g/kg body weight) given subcutaneously produced only benign tumors at the injection site and, hence, was not considered carcinogenic.

Bonser, Clayson, and Jull (1956) subcutaneously administered benzidine or 3,3'-dihydroxybenzidine in weekly doses of 6 mg for 52 weeks to mice until exitus. Benzidine induced hepatomas and lymphomas, whereas 3,3'-dihydroxybenzidine induced lymphomas and benign intestinal polyps. Induction periods were 70 weeks. One-third of the control animals developed spontaneous lymphomas, which made significant interpretation difficult. 3,3'-Dihydroxybenzidine was a weak carcinogen in mice, causing tumors of the liver and mammary gland and also causing leucosis when it was given subcutaneously (Pliss, 1961). When 3,3'-dihydroxybenzidine was applied to the skin, the mice developed tumors of inner organs. Benzidine administered subcutaneously in weekly doses of 6 mg to mice of line C3HA produced hepatomas in 31 of 46 mice (Prokofjeva, 1971). One mouse had a pulmonary adenocarcinoma. The first tumors appeared 15 to 16 months following the beginning of benzidine administration.

N,N'-Diacetylbenzidine produced tumors in female Wistar rats given a single intraperitoneal (100 or 200 mg) or subcutaneous (100 mg) injection (Bremner and Tange, 1966). In 14 of 30 rats, 24 tumors developed principally in the external auditory canals and mammary glands 6 to 15 months after injection. Interestingly, tumors did not develop in the 6 animals given 200 mg intraperitoneally, whereas they did develop in 11 out of 18 animals given 100 mg by the same route.

6.3.2.2.2 3,3'-Dimethylbenzidine (Diorthotolidine) — There is no evidence that diorthotolidine is carcinogenic in humans (Rye, Woolrich, and Zanes, 1970). It is a weaker carcinogen than benzidine in animals (Sax, 1975), and this difference may carry over to humans. Transient hematuria is a common symptom in workers exposed to diorthotolidine (Hamblin, as cited in Rye, Woolrich, and Zanes, 1970). Since diorthotolidine follows a metabolic pathway similar to benzidine (Rye, Woolrich, and Zanes, 1970), it should be treated as a potential industrial carcinogen (Sax, 1975).

The only data concerning diorthotolidine carcinogenicity are obtained from studies dealing with rats and mice. Rats treated with an accumulative dose of 5.4 g/kg body weight of diorthotolidine for 241 days developed 11 gastrointestinal tract tumors, 7 hepatomas, 4 tumors of bone and associated tissues, and 4 carcinomas of the Zymbal's gland. Twenty female Sprague-Dawley rats were given diorthotolidine orally up to a total dose of 500 mg per rat. At the end of nine months, 3 of 16 surviving rats had a total of four mammary carcinomas (Griswold et al., 1968). The method of administration seems to influence where tumors develop. Pliss and Zabezhinsky (1970) administered diorthotolidine to rats either by subcutaneous injection (20 mg per rat) or by subcutaneous implantation of pellets (20 mg per rat) for 13 to 14 months. Tumors, mainly of the skin, large sebaceous glands, and mammary glands, developed in 60% to 70% of the rats (Table 6.12). Implantation of pellets of diorthotolidine induced subcutaneous sarcomas in 2 of 68 rats and hepatocellular carcinomas in 4; this effect was not found when diorthotolidine was injected.

6.3.2.2.3 3,3'-Dimethoxybenzidine (Dianisidine) — Dianisidine is another benzidine derivative in which there is no evidence of carcinogenicity in humans (Rye, Woolrich, and Zanes, 1970). However, several studies have provided evidence that it is carcinogenic to rats. Dianisidine administered by subcutaneous injection induced tumors of the Zymbal's gland in two rats and an ovarian tumor and fibroadenoma of the mammary glands in another rat (Pliss, 1963). The incidence rate was low since only 3 of 18 rats developed tumors. After 260 doses of dianisidine (10 mg per dose) given orally, both male and female Fischer rats developed tumors in the gastrointestinal tract, skin, breast, and ear duct (Weisburger et al., 1967). Tumors developed as early as 293 days after the first administration.

6.3.2.2.4 3,3'-Dichlorobenzidine — No cases of bladder cancer in humans attributable to dichlorobenzidine have been reported (Rye, Woolrich, and Zanes, 1970). However, tumor induction by this compound has been demonstrated in rats and mice, and there is some evidence for its complicity in the occurrence of nonbladder tumors in humans.

TABLE 6.12. TUMORS INDUCED IN RATS RECEIVING 20 mg OF DIORTHOTOLIDINE EITHER BY SUBCUTANEOUS INJECTION OR SUBCUTANEOUS IMPLANTATION OF PELLETS

Method of administration	Number of rats at beginning of experiment	Time of first tumor appearance (months)	Number of rats		Number of rats with tumors at specific sites[a]						Subcutis at site of implantation	Other
			Survived to time of first tumor	With tumors	Zymbal's gland	Mammary gland	Skin	Preputial gland	Liver	Fore-stomach		
Injection	27 males	8	25	17	14 (8–22)		2 (18–23)	2 (20–22)	1 (23)	3 (21–25)		5[b]
	26 females	8	25	13	6 (8–20)	5 (13–22)	1 (16)	1 (13)				1[c]
	Total 53	8	50	30	20	5	3	3	1	3		6
Implantation	24 males	12	16	11	6 (12–18)		1 (17)		3 (15–20)		1 (20)	3[d]
	24 females	12	20	12	5 (12–18)	7 (12–23)	1 (18)					1[e] (20)
	Total 48	12	36	23	11	7	2		3		1	4

[a] Numbers in parentheses indicate time of tumor appearance in months.

[b] In one rat, tumor of small intestine; in two rats, tumors of hematopoietic system (in 12 and 23 months respectively); in one rat, thyroid tumor (in 25 months); in one rat, lung tumor (in 22 months).

[c] Uterine tumor.

[d] In one rat, angiosarcoma in area of anterior extremity (in 14 months); in one rat, neurosarcoma in area of the ear (in 15 months); in one rat, tumor of hematopoietic system (20 months).

[e] Lymphangioma in neck region.

Source: Adapted from Pliss and Zabezhinsky, 1970, Tables 1 and 2, p. 285.

Gerarde and Gerarde (1974) found no bladder tumors in employees at a New York plant of Hercules, Inc. The workers were exposed to dichlorobenzidine for 35 years. However, 17 workers developed neoplasms in other locations: 2 in the lung, 1 in bone marrow, 6 lipomas, 3 papillomas of the rectum, 2 carcinomas of the sigmoid colon, 1 prostate carcinoma, 1 myoblastoma of the breast muscle, and 1 basal cell epithelioma. At a British plant using dichlorobenzidine for 30 years, no cases of bladder cancer were found among more than 200 exposed employees (MacIntyre, 1975). Most of the exposed workers had been exposed for less than 16 years, which indicates that the latent period may not have been reached.

A comparison of tumor incidence was made between workers exposed to dichlorobenzidine and those exposed to both benzidine and dichlorobenzidine at a Clayton Aniline plant (Gadian, 1975). Workers exposed to benzidine plus dichlorobenzidine developed three bladder tumors, whereas those exposed to dichlorobenzidine alone did not develop any bladder tumors (Table 6.13). The employees had been exposed to dichlorobenzidine for 10 to 15 years, during which time occasional bladder tumor cases should have appeared if the latent period were similar to benzidine. It is possible, however, that the latent period is longer. If dichlorobenzidine is a human carcinogen, it would appear to be weaker than benzidine.

Dichlorobenzidine is carcinogenic to several animal species. Pliss (1963) injected 3,3'-dichlorobenzidine subcutaneously into rats and found tumors in 74% of the animals. Tumors developed in the skin, sebaceous and mammary glands, intestines, bones, and urinary bladder. Tumors developed in 48 of 64 D-line mice that survived to the appearance of the first tumor after receiving dichlorobenzidine by ingestion or injection into the underlying fat (Pliss, 1959). Sarcomas were found at the injection site in seven rats. Two rats ingesting the compound had adenocarcinomas of the small intestine. Tumors were found also in the mammary and sebaceous glands, and three rats had papillomas of the bladder.

Griswold et al. (1968) did not find any tumors in rats given dichlorobenzidine dihydrochloride (300 mg per rat total dose) by oral feeding. There was a significant increase in tumor number in progeny of BALB/c mice given dichlorobenzidine (8 to 10 mg per mouse total dose) by subcutaneous injection (Golub, Kolesnichenka, and Shabad, 1974). Tumors developed in 13 of 24 mice: 4 mice had adenocarcinomas of the mammary gland, 5 had adenomas of the lung, and 7 had lymphatic leukemia. Rats receiving 1000 ppm dichlorobenzidine in the diet had tumors of the mammary gland in both males and females and tumors in the Zymbal's gland and hematopoietic tumors in males (Stula, Sherman, and Zapp, 1971; Stula et al., 1975).

6.3.2.2.5 4-Aminobiphenyl — Melick et al. (1955) studied the workers in a plant manufacturing 4-aminobiphenyl from 1935 to 1955. Of the 171 exposed employees, 11.1% had developed bladder tumors from 5 to 19 years after initial exposure. The duration of exposure was 1.25 to 19 years. A metabolite of 4-aminobiphenyl was suspected as being the carcinogen. In a follow-up study to the Melick et al. (1955) study, bladder tumors were found in workers exposed to 4-aminobiphenyl in two plants manufacturing this compound (Melick, Naryka, and Kelly, 1971). The minimum exposure time to

TABLE 6.13. COMPARISON OF TUMOR INCIDENCE BETWEEN WORKERS EXPOSED TO DICHLOROBENZIDINE AND THOSE EXPOSED TO DICHLOROBENZIDINE PLUS BENZIDINE

Group	Number of men	Exposure (hr)	Tumor incidence		Deaths from other causes
			Urinary tract	Other	
Segregated dichlorobenzidine workers	35	68,505	None	None	One coronary thrombosis, one cerebral hemorrhage
Mixed benzidine and dichlorobenzidine workers	14	16,200[a]	One papilloma of bladder, two carcinomas of bladder	One carcinoma of bronchus	One coronary thrombosis

[a] Exposure to approximately 60% benzidine and 40% dichlorobenzidine.

Source: Adapted from Gadian, 1975, Table 1, p. 828. Reprinted by permission of the publisher.

tumor induction was 133 days; the longest time interval for tumor development to date was 35 years. The incidence rate of bladder tumors between 1957 and 1970 increased from 2.3% to 18.5% in one plant, probably because of workers reaching the latent period required for tumor development. A strong association may exist between bladder cancer and occupational exposure to 4-aminobiphenyl (International Agency for Research on Cancer, 1972).

Bladder tumors have been induced in dogs given 4-aminobiphenyl. Two beagle dogs administered 4-aminobiphenyl (30 to 34 g total) by mouth developed advanced bladder tumors and were killed 33 months after first dosing (Walpole, Williams, and Roberts, 1954). Since the tumors appeared early in the treatment relative to tumor induction time of benzidine, 4-aminobiphenyl was more effective than benzidine as a bladder carcinogen in dogs. All female beagle dogs fed 4-aminobiphenyl (0.66 to 0.74 g/kg body weight) for three years developed from one to several papillomas or carcinomas of the bladder (Deichmann et al., 1965). 4-Nitrobiphenyl was fed to beagle dogs at the same dose level, and at the end of the three-year period the bladders of all treated dogs were normal. 4-Aminobiphenyl was more carcinogenic than 4-nitrobiphenyl in the urinary bladder of dogs. Deichmann and MacDonald (1968) found that a single dose of 50 mg 4-aminobiphenyl per kilogram body weight administered by mouth to beagle dogs did not induce precancerous lesions of the bladder mucosa or tumors in the urinary bladder. Since the dose size was much smaller than that used by others in producing tumors, tumor development would not necessarily be expected.

4-Aminobiphenyl is carcinogenic in rats and mice, although the effective carcinogen is probably an *o*-hydroxyamine formed in the body's metabolic hydroxylation (Walpole, Williams, and Roberts, 1952). Rats subcutaneously injected with 4-aminobiphenyl at total dose levels of 5.8, 4.4, 4.2, and 3.6 g/kg body weight developed tumors of the liver, intestines, mammary glands, kidney, and uterus. Newborn male and female Swiss mice were given 200 μg of 4-aminobiphenyl by subcutaneous injection during the first three days of life (Gorrod, Carter, and Roe, 1968). At the termination of the study, 48 to 52 weeks after injection, greater than 90% of the male mice and about 18% of the females developed hepatomas. One pulmonary adenoma, one thymic lymphoma, and one lymphosarcoma of the spleen also were found. Testing three hydroxylated derivatives of 4-aminobiphenyl (4-amino-3-hydroxybiphenyl, 4-hydroxylaminobiphenyl, and 4-amino-4'-hydroxybiphenyl) in the same manner produced a significant increase of hepatomas in males above the controls and a probable significant increase of hepatomas in females in response to three of the compounds, but not to 4-amino-3-hydroxybiphenyl.

SECTION 6

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SECTION 7

ENVIRONMENTAL DISTRIBUTION AND TRANSFORMATION

7.1 SUMMARY

Benzidine and its related compounds (3,3'-dichlorobenzidine, 3,3'-dimethylbenzidine, and 3,3'-dimethoxybenzidine) are used primarily as intermediates in the production of azo dyes. Additionally, they are used in polyurethane production and in chemical analyses.

Benzidine is not thought to be commonly distributed in the environment. In water, it is a probable hazard only near dye factories. Water samples collected below Japanese dye factories have contained up to 0.233 ppm benzidine. No data concerning benzidine in air and soil were found.

Information regarding degradation of benzidine under normal environmental conditions is not available, except for one survey of the Buffalo and Niagara river areas. Laboratory studies indicated that benzidine tends to resist biological and physical degradation. Estimated half-lives for benzidine are 1 day for reaction with OH or O_3 in air and 100 days for reaction with RO_2 in water.

Based on its solubility in water and various organics, benzidine is suspected to accumulate within food chains. However, no specific data are available other than a laboratory study indicating bioaccumulation in bluegill fish to a factor of 44 times the level in water.

7.2 PRODUCTION AND USE

7.2.1 Benzidine

Benzidine is used primarily as an intermediate in the production of azo dyes. It is also used as a hardener for polyurethane, in qualitative and quantitative chemical analyses, in the detection of occult blood, as a microscopy stain, and as an agent to reveal bank check alteration (International Agency for Research on Cancer, 1972; Lurie, 1963; Sciarini, 1969; Welcher, 1947). Benzidine production in the United States was 680.9 metric tons (750 tons) in 1972 (Anonymous, 1974).

7.2.2 3,3'-Dichlorobenzidine

3,3'-Dichlorobenzidine, a dye intermediate and curing agent for polyurethane, gives azo dyes which are bluer and more stable to acids than those from benzidine (Radding et al., 1975). More than 95 dyes are derived from 3,3'-dichlorobenzidine (*Colour Index*, 1957); the primary one is benzidine yellow (Hancock, 1975). United States production of 3,3'-dichlorobenzidine and its salts was 1,600,000 kg (3,500,000 lb) in 1971 (U.S. Tariff Commission, 1973, cited in International Agency for Research on Cancer, 1974). Imports in 1971 were 658,000 kg (1,450,890 lb) (International Agency for Research on Cancer, 1974).

7.2.3 3,3'-Dimethylbenzidine

More than 90 dyes are derived from 3,3'-dimethylbenzidine (diorthotolidine) (*Colour Index*, 1957). Additionally, diorthotolidine is used in detection of various ions (Welcher, 1947). United States production was approximately 110,200 kg (243,000 lb) in 1962 (Lurie, 1964). In 1969 and 1970, U.S. imports amounted to 36,600 kg (80,600 lb) and 44,400 kg (97,800 lb) respectively (International Agency for Research on Cancer, 1972).

7.2.4 3,3'-Dimethoxybenzidine

3,3'-Dimethoxybenzidine (dianisidine) is used primarily in dye production, with 88 dyes derived from this chemical (*Colour Index*, 1957). It is also used to detect copper, cobalt, gold, thiocyanate, and vanadium (Welcher, 1947). The United States produced 163,000 kg (360,000 lb) of dianisidine in 1960 (U.S. Tariff Commission, cited in Lurie, 1964).

7.3 DISTRIBUTION IN THE ENVIRONMENT

7.3.1 Distribution in Soils

No data concerning benzidine measurements in soils were located in the literature. Due to its physical properties, benzidine probably is immobilized rapidly in soils and sediments (Radding et al., 1975). Furukawa and Brindley (1973) found that benzidine was adsorbed to some clays.

7.3.2 Distribution in Water

Benzidine in water is probably a hazard only in the vicinity of dye and pigment factories from which wastes escape or are discharged. Generally, processes in azo dye production are conducted within a closed system. Contamination can occur, however, during cleanup (Haley, 1975).

Howard and Saxena (1976, pp. 16-17) reported on a field survey made by the Synthetic Organic Chemical Manufacturers Association Task Force on Benzidine. The survey was made to determine whether benzidine could be detected in the Buffalo and Niagara river areas, where some benzidine release by upriver plants had been thought to occur. No benzidine was detected in either water or sediment samples when they were analyzed by the chloramine-T method, which had a limit of detection of 0.2 μg/ml (for 1-liter water samples). As pointed out by the authors, lack of detection of benzidine in these samples may have resulted either from dilution to below the limit of detection or from oxidative degradation into compounds not detectable by this method of analysis.

Benzidine was detected (samples containing 0.082, 0.140, and 0.233 ppm) in the Sumida River (Japan) below dye factories (Takemura, Akiyama, and Nakajima, 1965). These authors speculated, however, that the benzidine may not have been discharged as such but was produced by reduction of azo dyes in the wastes by H_2S or SO_2 present in the river.

7.3.3 Distribution in Air

Although benzidine in closed air systems is potentially hazardous (Zavon, Hoegg, and Bingham, 1973), no data were located concerning its distribution in the atmosphere. The azo dye benzidine yellow, derived from 3,3'-dichlorobenzidine, is used in various paints, enamels, and lacquers and has been found in pressurized containers designed for the hobbyist and householder market. Such usage in the home and work environment may pose a hazard to the user as well as provide an avenue of air contamination and dispersion.

7.4 ENVIRONMENTAL FATE

7.4.1 Mobility and Persistence

Benzidine is volatile and soluble enough to have a potential for wide dispersion. According to several authors, benzidine resists physical and biological decomposition (Lutin et al., 1965; Malaney et al., 1967; Radding et al., 1975) and probably persists for a time in the environment. The principal chemical reaction of benzidine in air and water is oxidative degradation by free radical, enzymatic, or photochemical processes (Radding et al., 1975). Data concerning the reaction rate of benzidine with radicals and ozone and on photochemical reactions under normal environmental conditions are not available. However, estimated half-lives are 1 day for reaction with OH or O_3 in air and 100 days for reaction with RO_2 in water (Radding et al., 1975).

In a study of benzidine degradation under experimental water treatment conditions, Howard and Saxena (1976, pp. 16-17) noted that air oxidation of benzidine seemed to have occurred readily and that some biological oxidation may also have occurred.

3,3'-Dichlorobenzidine is thought to be bound tightly to humic materials (Radding et al., 1975). Due to the halogen substitution, 3,3'-dichlorobenzidine probably degrades more slowly than benzidine. Its estimated half-lives are 1 day with OH radicals, 1 to 10 days with O_3, and 100 days with RO_2 radicals.

7.4.2 Accumulation in Food Chains

Benzidine and 3,3'-dichlorobenzidine are more soluble than DDT in water and are quite soluble in many organics (Sections 2.2.1.1 and 2.2.2.1). Therefore, it is suspected that these chemicals can move within the food chain. Accumulation is expected to occur, and recent laboratory findings indicated that bluegill fish concentrate residues of ^{14}C-benzidine to a level 44 times that present in water (*Federal Register*, 1976).

SECTION 7

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SECTION 8

ENVIRONMENTAL ASSESSMENT

8.1 PRODUCTION, USES, AND POTENTIAL ENVIRONMENTAL CONTAMINATION

8.1.1 Production

Although exact figures for current U.S. production and importation of benzidine and benzidine compounds are unavailable, an examination of records of the U.S. Tariff Commission for past years indicates that U.S. production and importation of these substances may exceed 3.5 million kilograms per year. In 1972, benzidine production was 680,000 kg, while that of dichlorobenzidine was 2,300,000 kg in 1971. Records for the 1960s indicate that total production and importation of 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine ranged from 200,000 to 500,000 kg per year.

8.1.2 Uses

Benzidine and its congeners are used in industry primarily for the synthesis of azo dyes. More than 250 dyes are derived from benzidine, 95 from dichlorobenzidine, 90 from 3,3'-dimethylbenzidine, and 88 from 3,3'-dimethoxybenzidine. The leather, paper, and textile industries utilize more than 10 million kilograms of such dyes every year. In addition, benzidine, 3,3'-dimethylbenzidine, and 3,3'-dimethoxybenzidine are widely used in chemical and biochemical laboratories as analytical reagents. Dichlorobenzidine and 3,3'-dimethoxybenzidine are also important in the polyurethane industry — the latter compound for the production of 3,3'-dimethoxy-diisocyanates and the former as a curing agent.

8.1.3 Losses to the Environment

There is little published information available to indicate the extent to which benzidine, benzidine congeners, and benzidine-based dyes are inadvertently released into the environment. The manufacturing and processing operations for benzidine salts and azo dyes are now contained within closed systems; however, some losses to the environment undoubtedly still occur, especially during cleaning operations.

In the past, in the absence of evidence that any of these compounds might be health hazards, routine sampling for air or water contamination was not practiced. As epidemiological data began to suggest that benzidine might be a human carcinogen, a greater effort was made to determine levels of atmospheric contamination at benzidine manufacturing plants. Several studies revealed that atmospheric concentrations of benzidine in such plants normally ranged from 0.002 to 0.152 mg/m^3 (Meigs, Sciarini, and Van Sandt, 1954; Zavon, Hoegg, and Bingham, 1973) and could reach as high as 17 mg/m^3 (Zavon, Hoegg, and Bingham, 1973).

Since 1974, when more stringent industrial standards were instituted by the Occupational Safety and Health Administration (OSHA) of the U.S. Department of Labor (*Federal Register*, 1974), the risk of cancer from industrial exposure to benzidine has been substantially reduced. Although the OSHA standards did not recognize a maximum safe level of exposure, they did attempt to reduce exposure "to the maximum extent possible consistent with continued use." Mixtures of compounds containing less than 0.1% of benzidine or its salts were excluded from the regulation in an attempt to avoid hindering scientific research on these substances. However, this exclusion clause in effect permitted a 0.1% level of contamination in compounds, such as various azo dyes, which are produced from benzidine. Currently, dye manufacturers warrant their products to contain not more than a certain level of free benzidine. This level (in one case 20 ppm) is generally much lower than the OSHA standard; however, it still represents a potential source of environmental contamination. Consequently, traces of benzidine salts probably occur in the wastewater effluents of the textile, paper, and leather manufacturing plants using azo dyes. Such manufacturing plants generally use several gallons of dye per pound of fabric processed, and the total amount of wastewater generated is often more than one million gallons per day.

There is increasing evidence that azo dyes, some of which may be toxic themselves, are capable of being reconverted by chemical or biological mechanisms into the toxic parent compounds (Rinde and Troll, 1975; Takemura, Akiyama, and Nakajima, 1965; Yoshida and Miyakawa, 1973). It may therefore be very important to determine the extent of environmental contamination by azo dyes. Published information on this subject is, however, quite limited.

In the azo dye plants themselves, removal of benzidine contaminants from wastewater effluents is sometimes accomplished by tetrazotization. Whether this process leads to a safe degradable end product or whether benzidine or some other toxic substance might be resynthesized by later chemical or microbial processes has not been fully examined. However, the question is important in light of the fact that benzidine wastes are often piped directly into municipal sewage systems.

8.2 ENVIRONMENTAL PERSISTENCE

8.2.1 Physical and Chemical Degradation

Very little information is currently available on the environmental fate of benzidine, its congeners, or benzidine-based dyes. Although benzidine is a moderately volatile substance, naturally occurring photochemical and chemical processes would probably reduce atmospheric concentrations to insignificant levels; however, the rates of such reactions are not well known. Benzidine is sensitive to various oxidative processes. With O_3 or HO radicals the half-life of benzidine in a gaseous state has been calculated to be about one day (Radding et al., 1975), and that of dichlorobenzidine is about ten days.

If there should prove to be any environmental accumulation of benzidine or its congeners, it might be expected that this would occur in natural bodies of water receiving effluents from manufacturing and processing plants.

According to Radding et al. (1975), the half-lives of benzidine and dichlorobenzidine in an aqueous state would be about 100 days if the compounds reacted primarily with RO_2 radicals. However, because the RO_2 rate constants used for these calculations were only estimates, the actual half-lives may be as little as 10 days or as much as 1000 days. With a relatively long half-life, the processes of biological degradation may be more important in reducing benzidine levels in aquatic environments. Because of its halogen substitution, dichlorobenzidine may be more resistant to biodegradation (Radding et al., 1975).

The solubility of the benzidine compounds would also have a significant effect on their environmental fate. Although benzidine is only slightly soluble in water (0.52 mg/ml at 25 ± 2°C), its chloride salts, which are used quite extensively in the azo dye industry, are very soluble in water (61.7 mg/ml) (Bowman, King, and Holder, 1976). In a similar manner, the benzidine congeners 3,3'-dimethylbenzidine and 3,3'-dimethoxybenzidine are much less soluble (1.3 and 0.06 mg/ml respectively) than their corresponding chloride salts (76.7 mg/ml and 41.4 mg/ml).

The chloride salt of benzidine has also been reported to be more resistant to degradation than the free amine. Bowman, King, and Holder (1976) found that over a 16-day period, the concentration of an aqueous solution of benzidine declined 11% while that of its salt declined only 2%. The concentration of both 3,3'-dimethylbenzidine and its salt declined 9% during the test period, and that of 3,3'-dimethoxybenzidine and its salt declined 64% and 9% respectively.

8.2.2 Biodegradation

It is known that benzidine and its congeners undergo in vivo oxidation in higher organisms; however, there is also evidence that benzidine is resistant to decomposition by microorganisms. Lutin, Cibulka, and Malaney (1965) and Malaney et al. (1967) found that benzidine at concentrations of 500 mg/liter was usually inhibitory or even toxic to sludge microorganisms, thus preventing normal biodegradative processes. The studies of Jenkins and Baird (1975) suggest that the toxic substance may not have been benzidine itself, but a reaction product resulting from chlorination. These investigators found that in unchlorinated wastewater samples spiked with benzidine 70% to 95% of the chemical could be removed analytically, but in chlorinated samples only a very small amount could be recovered. This suggested that benzidine reacted with hypochlorous acid to produce a chloramine-type compound. Chloramines are known to be toxic to a great number of aquatic organisms.

It has been reported by several investigators that some azo dyes can be reduced to free benzidine by soil and intestinal bacteria (Yoshida and Miyakawa, 1973). Presumably, similar biochemical transformations occur in bacteria in aquatic systems.

Numerous nonbenzidine azo food dyes are known to undergo reduction to various metabolites by way of the hepato-azo reductase system of higher organisms (see Walker, 1970, for a recent review). Metabolic reduction of benzidine dyes to benzidine has recently been demonstrated in the rhesus monkey (Rinde and Troll, 1975), and benzidine dyes have been implicated as carcinogenic agents in painters accidently ingesting these dyes (Yoshida and Miyakawa, 1973).

8.3 EFFECTS ON AQUATIC AND TERRESTRIAL ORGANISMS

8.3.1 Nonlaboratory Organisms

Although there is a substantial amount of information on the effects of benzidine on standard laboratory research animals such as rats, mice, and dogs, very little is known about the toxicity of benzidine to other kinds of organisms. That benzidine can be toxic to some plants and wild animals is shown by the studies of Fitzgerald, Gerloff, and Skoog (1952) and Pliss and Khudoley (1975). In analyzing the toxicity of several organic compounds to the blue-green alga *Microcystis aeruginosa*, Fitzgerald, Gerloff, and Skoog (1952) found that benzidine, at concentrations of 20 mg/liter, was lethal to 50% of five-day algal cultures. Pliss and Khudoley (1975) examined the effects of benzidine on the freshwater guppy. They found that although the chemical did not produce tumors in the fish, its toxic effects included extensive liver damage. Since the benzidine dosage used by these investigators was calculated in terms of food intake (30 mg of benzidine per 100 g of dry food), there is no way to correlate these results with potential benzidine concentrations in natural bodies of water.

8.3.2 Laboratory Animals

The toxic effects of benzidine and its congeners have been found to depend on several factors: (1) physiochemical form of the compound administered, (2) size of the dose, (3) mode of administration, (4) length of exposure, and (5) species of animal undergoing the tests. In most species tested these substances tended to cause the greatest damage to the liver, kidney, and urinary bladder. The gall bladder, intestines, and lymph glands can also be affected to a lesser extent. Toxic levels of benzidine often lead to extensive liver damage. In many laboratory studies, excessive deaths due to the poisoning have obscured the carcinogenic potential of these compounds. However, following administration of sublethal doses tumors have formed in almost all species studied. In various species of rodents, hepatomas, lymphomas, and adenocarcinomas have resulted from exposure to benzidine and 3,3'-dihydroxybenzidine. In contrast, in hamsters, rabbits, and dogs the carcinogenic activity of benzidine is centered almost solely in the bladder. The benzidine congeners are also known to be carcinogenic in rats and mice; the target organs depend considerably on the mode of administration and concentration of the compound. Tumors have been produced in the rat and mouse in the skin, gastrointestinal tract, liver, bone, ovaries, Zymbal's gland, sebaceous glands, mammary glands, and urinary bladder.

8.4 EFFECTS ON HUMAN HEALTH

8.4.1 Toxic Effects

Schwartz, Tulipan, and Peck (1947) have reported on the occurrence of dermatitis among workers in azo dye manufacturing plants. In several instances it was found that the dermatitis resulted from exposure to benzidine or benzidine-based dyes. Laboratory exposure to 3,3'-dimethylbenzidine, as well as to benzidine, has also been known to cause dermatitis among chemists. Hematuria occasionally occurs in workers exposed to 3,3'-dimethylbenzidine or benzidine, and the latter compound is also known to cause chronic cystitis.

8.4.2 Teratogenic Effects

There is no evidence that benzidine or its congeners act as human teratogens. In one of the few laboratory studies having any possible relevance to this subject, Noto (1967) found that albumen suspensions of benzidine (3 mg) injected into a chicken egg resulted in morphological abnormalities in the developing embryo. The chemical was thought to inhibit morphogenetic movements, thus causing the unclosure of the neural tube. Other known carcinogens such as 4-nitroquinoline-N-oxide and *o*-aminoazotoluene caused similar abnormalities.

8.4.3 Mutagenic Effects

Ames et al. (1973) proposed that many known carcinogens, including benzidine, owe their carcinogenic activity to the fact that they cause somatic mutations. In tests using mutant strains of the bacteria *Salmonella typhimurium* and a rat or human liver homogenate to provide the necessary enzyme systems responsible for activating the carcinogen, these workers found that benzidine caused a significant increase in the number of revertant bacteria colonies. The carcinogen was thought to have caused frameshift mutations involving addition or depletion of DNA base pairs. Such a change in the structure of DNA might produce an inheritable change in cell regulation which could thereby lead to the formation of a tumor.

8.4.4 Carcinogenic Effects

Epidemiological evidence leaves no doubt that exposure to benzidine can lead to bladder cancer in humans. Although there is no evidence to indicate that the benzidine congeners 3,3'-dimethylbenzidine, 3,3'-dimethoxybenzidine, or 3,3'-dichlorobenzidine can cause bladder cancer in humans, all three compounds have been implicated as carcinogens in animal studies. Tumors of other human organ systems (intestinal tract, lungs, bone, and muscles) have been found in some dichlorobenzidine workers (Gerarde and Gerarde, 1974), but the significance of this finding has not yet been established.

Various studies have shown that the actual chemical substance causing bladder cancer following benzidine exposure is not benzidine itself but a metabolic by-product of the compound. Enzyme systems that are found primarily in the liver presumably convert benzidine into N-hydroxylated derivatives which may then require esterfication to become further activated. The ultimate carcinogens may be sulfate or glucuronidate conjugates of the metabolically altered parent compound (see Section 2.2.1.5).

An exact quantitative relationship between the degree of exposure to benzidine, the length of the exposure period, and the incidence of bladder cancer has not been established. Studies with laboratory animals are not very indicative in this regard; there appears to be species-specific differences in the relative toxicity and carcinogenicity of the compounds, rate and route of uptake and excretion, and metabolic pathways. Studies at benzidine manufacturing plants indicated that workers had been exposed to atmospheric concentrations of benzidine from 0.002 to 17.3 mg/m^3 (Meigs, Sciarini, and Van Sandt, 1954; Zavon, Hoegg, and Bingham, 1973), yet there is no way of accurately estimating the level of exposure and the amount taken up by individual workers. Laboratory studies on animals led Meigs, Sciarini, and Van Sandt (1954) to speculate that approximately 5% of the amount of benzidine taken up is excreted as the free amine. Presumably the remaining 95% undergoes metabolic transformation. Whether all the by-products are excreted or whether some might be stored in tissues has not been shown. The degree to which in vivo oxidation renders the compounds harmless is also unknown.

According to Zavon, Hoegg, and Bingham (1973) there is a nonlinear relationship among exposure levels, exposure periods, and incidence of bladder tumors. Supposedly, a minimum exposure will be followed by a prolonged latent period, but increased exposure will not necessarily result in decreased latency or increased incidence of the disease (Scott, cited in Zavon, Hoegg, and Bingham, 1973). This hypothesis would seem to be confirmed by the fact that the latent period is quite prolonged (average time 16 to 20 years) in benzidine workers developing bladder tumors, regardless of exposure histories.

Meigs, Sciarini, and Van Sandt (1954) calculated that an atmospheric concentration of 0.02 mg/m^3 might be safe for industrial workers. They based this assumption on the fact that workers exposed to this level of benzidine (estimated from the concentration of benzidine in their urine, which was thought to be 5% of the amount taken up) were reportedly unlikely to develop bladder tumors, whereas workers with higher exposures were more susceptible. The validity of these calculations has not been verified by other investigators.

8.5 POTENTIAL HEALTH HAZARDS

8.5.1 Industrial Workers

Since the late 1950s when it was definitely established that benzidine was carcinogenic, there has been a general effort to reduce the levels of exposure to which benzidine workers are subjected. In 1974 the U.S. Department of Labor announced specific regulations governing the conditions under which benzidine and the benzidine congeners could be manufactured and processed (*Federal Register*, 1974). Presumably, these regulations have reduced benzidine concentrations in industrial environments to safe levels; however, these regulations do not recognize maximum permissible levels of contamination. In the absence of published information on the current monitoring practices in the benzidine industry, of data regarding current levels of exposure, and of a proven minimum exposure threshold, it is difficult to evaluate current health hazards in industry; however, the reduction in tumor incidence would suggest that the risks for benzidine workers have been substantially reduced in recent years.

8.5.2 Laboratory Workers

A number of reports have emphasized the potential dangers of benzidine and the benzidine congeners in chemical, biochemical, and microbiological laboratories where these compounds are often used as analytical reagents (Collier, 1974; Veys, 1972; Wood and Spencer, 1972). Levels of contamination would undoubtedly vary from laboratory to laboratory, and it is very likely that a broad epidemiological study of laboratory workers would be necessary to determine if laboratory exposure to these compounds constitutes a significant hazard. In view of the potential danger, suitable alternative analytical reagents should be recommended, and if no such alternatives exist, users of these compounds should be adequately warned of their potential carcinogenicity.

8.5.3 General Population

There is no evidence that benzidine or any of its congeners represent a health hazard to the general public. The little information that is available suggests that any benzidine released into the atmosphere would undergo rapid physiochemical degradation. It is more likely that if dangerous environmental levels of benzidine are found, these would be restricted to water supplies near manufacturing and processing plants. However, these compounds have not been reported in any natural bodies of water in the United States. Whether this negative evidence is due to a lack of adequate monitoring data or to a real absence of these substances is unknown. It is quite possible that biological decomposition in water may proceed at a rapid rate; however, it has also been shown that benzidine is resistant to biodegradation in water treatment plants — possibly due to the formation of toxic chloramines. The contamination of potential water supplies is a distinct possibility as is indicated by a Japanese study showing that benzidine levels were quite high in a river downstream from a series of benzidine dye plants (Takemura, Akiyama, and Nakajima, 1965). This also raises the question as to whether environmental contamination by benzidine-based dyes is a greater potential hazard than that associated with benzidine alone. It has been reported that such dyes can be broken down into benzidine by soil and intestinal bacteria (Yoshida and Miyakawa, 1973); it is very likely that bacteria normally found in natural bodies of water would have the same capability. Benzidine dyes are used in a wide variety of commercial products, but as in the case of benzidine, there is very little information on the amounts being released into the environment.

8.6 POTENTIAL ENVIRONMENTAL HAZARDS

It has been shown that benzidine is potentially toxic to microorganisms, a species of blue-green algae, one species of freshwater fish, and various laboratory animals. Although the data are not very extensive, they lead to the conclusion that sufficiently high concentrations of benzidine may prove to be disruptive to the environment. In view of the fact that the most likely route of entry into the environment would be through industrial effluents, aquatic systems may be the most susceptible to damage. There is, however, no evidence that toxic levels of benzidine or any benzidine compound are present in any natural bodies of water in the United States. This lack of evidence may be due to an insufficient amount of environmental monitoring data resulting from the general assumption that these substances are not environmental hazards. More refined analytical techniques are currently available to detect levels of contamination as low as 0.2 μg/liter and these should be used to determine whether any significant environmental contamination is occurring.

Although it would appear that benzidine lacks the physiochemical characteristics — such as resistance to chemical and biological degradation — to make it a major long-range environmental problem, its toxicity and carcinogenicity are sufficient to require that a continuing effort be made to evaluate its effects on the environment.

8.7 REGULATIONS AND STANDARDS

The industrial standards instituted by OSHA in 1974 excluded from regulation any compounds containing less than 0.1% benzidine. These standards did not recognize a safe level of air or water contamination, nor were any provisions made for environmental monitoring.

In 1973 the Environmental Protection Agency proposed a toxic pollutant standard for benzidine which was based primarily on an extrapolation of animal test data (*Federal Register*, 1973). The standard proposed limited the amount of benzidine discharged into navigable waters to 1.8 μg/liter provided there was an immediate 1:10 rate of diffusion or to 0.18 μg/liter in situations where there was a slower rate of diffusion. Because of the limited data base, these standards were not promulgated.

In 1976 EPA proposed new standards for benzidine discharges (*Federal Register*, 1976). These standards were supported by evidence presented in *Criteria Document: Benzidine* (U.S. Environmental Protection Agency, 1976), which contained information on toxicological and environmental effects and fate of benzidine. These standards, which were promulgated in 1977 (*Federal Register*, 1977), establish an ambient water criterion for benzidine of 0.1 μg/liter. Effluent standards were set at 10 μg/liter (daily average) with a maximum for any single day of 50 μg/liter. Based on a monthly average, daily loading was limited to 0.130 kg per 1000 kg of benzidine produced. The standards set for users of benzidine-based dyes were the same except that the maximum daily effluent concentration of benzidine was limited to 25 μg/liter.

SECTION 8

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